TRANSFORMATION OF TYPE IN THE PNEUMOCOCCUS GROUP

BY

A. LANGVAD-NIELSEN

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A. LANGVAD-NIELSEN

1943

NYT NORDISK FORLAG
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Denne Afhandling er af det lægevidenskabelige Fakultet antaget til offentlig at forsvares for den medicinske Doktorgrad.

København, den 30. September 1943.

E. Rydberg,
h. a. dec.

NORDLUNDES BOGTRYKKERI, KØBENHAVN
18608

To My Wife

Translated from Danish by
Hans Andersen, M. D.

PREFACE

The work here presented was carried out in the State Serum Institute, Copenhagen, during my service as Assistant in the Pneumococcus Department from May 1940 to February 1942.

I wish to give my best thanks to the director of this institute, J. Ørskov, M. D., for his kind interest in my work and for the ideal working conditions offered me.

I am particularly indebted to F. Kauffmann, M. D., Chief of the Pneumococcus Department for his stimulating instruction and invaluable help in the course of this work, from its very start to its conclusion.

Further, I am greatly obliged to my colleagues and to the technical assistants in this department who in various ways have helped me in the performance of this work.

Last, not least, I wish to thank my wife, Eva Langvad-Nielsen for her valuable assistance in the performance of the experiments and in the proof-reading of this book—in particular for her inciting encouragement to carry through this work.

In conclusion I wish to acknowledge that the present studies were carried out in part with the aid of a grant from P. Carl Petersens Fond.

September 1943. *A. Langvad-Nielsen.*

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INRODUCTION

The important problem of transformation of types within the pneumococcus group, first described by F. Griffith and subsequently confirmed by some authors and disputed by others, is of such great scientific significance that it would seem reasonable to submit it to further investigation. The necessity of such investigation is the more urgent as so far no absolutely sure proof of a real transformation has been furnished.

The original experiments of F. Griffith consisted in simultaneous subcutaneous injection into mouse of non-capsulated pneumococci (R form, rough)—*e. g.*, Type 1—and heat-killed pneumococci (S form, smooth), *i. e.*, vaccine of another type—for instance, Type 2. If the experiment turns out successful, which is not always the case, the mouse dies of a pneumococcus infection due to capsulated pneumococci of the same type as the type of the vaccine, *i. e.*, Type 2. This means that the living type (R form) has been transformed into Type 2 (S form):

$$\text{R } 1 + \text{vaccine } 2 = \text{S } 2.$$

Naturally, the main objection to such a transformation type will be: that the vaccine has not been sterile (the vaccine was heated to 60° for 20 min.). Indeed, several authors—*e. g.*, Dunlop—have tried to prove that the vaccines employed in these experiments were not perfectly sterile.

On the other hand, the serological identity of the new-formed pneumococcus type obtained in this way with the type of the

vaccine was not ascertained indisputably by cross-absorption tests, so that the scepticism concerning these experiments really was not unreasonable.

At the suggestion of F. Kauffmann, I have therefore taken up this question for further investigation, for the purpose of proving with absolute certainty the correctness of the transformation of types described by F. Griffith—or eventually disproving it. I have to admit that here in the institute we were rather sceptical concerning this question, and at the commencement of the experiments we had our strong doubts about the asserted transformation of types.

PART I

HISTORICAL SURVEY

1. *Serological and Cultural Pneumococcus Types.*

Because of their thorough studies in 1884-86 on the morphology, isolation, cultivation and pathogenecity of the pneumococci for man and animals, *Fraenkel* (31-33) and *Weichselbaum* (88-89) are generally stated to have discovered this microorganism even though other investigators had seen it previously (*e. g., Klebs* (51), *Eberth* (30), *Mátray* (58-59), *Pasteur* (70-71), *Sternberg* (81), *Friedländer* (34)).

In 1909 and 1910 *Neufeld & Händel* (65-67) were able by means of serological methods to demonstrate the existence of several pneumococcus types, one of which was by far the most common and, therefore, designated by the authors as Type 1 or typical, while all the others were called atypical. Other investigators established and extended the observations reported by *Neufeld & Händel—e. g., Dochez & Gillespie* (28) (1913), *Avery* (6) (1915), *Stillmann* (83) (1919), and, in particular, *Cooper, Mishulow & Blanc* (15) (1921), *Cooper, Edwards & Rosenstein* (14) (1929) and *Cooper, Rosenstein, Walter & Peizer* (16) (1932) who differentiated 32 serologically different pneumococcus types.

Since 1932 extensive studies on new pneumococcus types have been reported by *Kauffmann, Mørch & Schmith* (49) (1940), *Walter, Guevin, Beattie, Cotler & Bucca* (87) (1941) and *Mørch* (61) (1942), so that now altogether 68 different pneumococcus types have been demonstrated.

In 1931 *Neufeld & Etinger-Tulczynska* (64) reported a new method for the diagnosis of pneumococci, which has since been

called the Neufeld reaction and now is employed almost exclusively for the typing of pneumococci. This method is based on an observation made by *Neufeld* (63) in 1902 in studies on the agglutination of pneumococci, as he found a distinct swelling of the pneumococcal capsule when a pneumococcal culture was mixed with homologous serum.

For details concerning the discovery of the pneumococci, their serological typing and type diagnosis, the reader is referred to *Erna Mørch*'s (62) dissertation.

Previously pneumococci were identified by employment of their capacity for fermentation of certain sugars and alcohols under acid formation, and especially the behavior of pneumococci towards inulin was the subject of investigation. Some authors—for instance, *Hiss, Borden & Knapp* (42), *Avery* (6), *Avery, Chickering, Cole & Dochez* (7), *Cooper, Mishulow & Blanc* (15)—asserted that the ability of pneumococci to ferment inulin was characteristic and an important diagnostic criterion, whereas others—*e. g., Levy* (57), *Christiansen* (13), *Cotoni, Truche & Raphael* (18) claimed that fermentation of inulin was not serviceable as a differential-diagnostic criterion.

Hiss, Borden & Knapp (42) (1905) investigated the fermentation power of pneumococci with employment of 11 sugars. *Christiansen* (13) (1913) studied the behavior of several pneumococcus types towards 22 sugars, polyvalent alcohols and glucocides.

Neufeld & Schnitzer (69) stated (in the textbook of Kolle, *Kraus & Uhlenhuth* (1928)) that it is characteristic of pneumococci that they are able to ferment saccharose, maltose, lactose, inulin and starch.

More comprehensive investigations on the fermentation power of the pneumococci have been carried out by *Viktorow, Semzowa & Sinjuschina* (86) (1933), who examined 88 strains of Types 1-4, and by *Jürgens* (46) (1937) who examined 122 pneumococcus strains of Types 1-3 and Group X. These authors found that all the strains examined were capable of fermentation under acid formation of d-glucose, d-mannose, d-galactose, d-fructose, lactose, saccharose, maltose, trehalose, raffinose, dextrin, glucogen and amygdalin, whereas they did not ferment d- and l-arabinose, xylose, l-rhamnose, erythrite, adonite, arabite,

dulcite, d-sorbite, perseite, inosite and arbutin. Further, they found no constant fermentation of inulin, mannite, melibiose, salicin and lactositol.

2. *Dissociation of the Pneumococci.*

The constancy of the biological character of the pneumococci depends on the surroundings. Under favorable conditions the morphology and serology of these bacteria will be constant; under unfavorable physical, biochemical or nutritional conditions, the properties of the pneumococci undergo certain changes manifest in their quality as to form and function. The virulence may be attenuated, and this will reduce the pathogenecity—even to complete lack of infectivity. The capacity for capsule formation may decrease or disappear completely, and the antigenic property of the pneumococci loses its strict type specificity, preserving merely the species specificity.

In the study of dissociation of the pneumococci, a great variation is observed in the deviating forms which are intermediate between the typical virulent forms and the entirely degraded atypical and avirulent forms.

These degraded pneumococci or variants may completely recover their original typical, morphological, cultural and immunologic properties if the injurious agencies cease and have been only of brief duration, and if the process of degradation has not been too violent. The regeneration may be effectuated either by letting the variants pass through an animal sensitive to the pneumococci—as a rule, white mice are employed—or by cultivation in suitable media, preferably containing antiserum produced by immunization with the degraded form, or by subcutaneous injection on white mice together with heat-killed virulent cultures of the homologous type.

The variability of the pneumococci was realized early. Thus, in 1892 *Kruse & Pansini* (53) described the changes in the morphology, cultural characteristics and virulence of pneumococci on artificial cultivation.

Arkharow (4) (1892) and *Issaef* (43) (1893) mentioned various changes in pneumococci which were cultivated in serum from

vaccinated animals or serum from animals which had died from pneumococcus infection. The growth of the pneumococci became more slow; in the sediment of the tube or along its walls they grew in long chains, and they were less virulent than pneumococci cultivated in ordinary serum broth.

Neufeld (63) (1902) found that pneumonococci cultivated in immune serum lose their virulence and, in connection herewith, their specific agglutinability and capacity for capsule formation.

During the following years, only slight interest was taken in the dissociation of the pneumococci, and this subject was not taken up for further investigation till 1915—by *Friel* (35) who cultivated various bacteria, including pneumococci, in immune serum, and found that the pneumococci were changed so as to become agglutinable and more sensitive to phagocytosis in normal serum.

In a comprehensive work, in 1916, *Laura Stryker* (84) reported the results she had obtained by cultivating pneumococci in 10 % immune serum. The agglutinability of the pneumococci was altered, the type specificity being lost; the longer a strain was grown in homologous serum, the more agglutinable it became in the heterologous serum and sometimes in normal serum; the virulence of the immune-serum-treated pneumococci for mouse and rabbit was decreased, and the capsule disappeared at the same time. The phagocytosis was increased, as the pneumococci now could undergo phagocytosis in normal horse serum as well as in immune serum.

Also the antigenic properties of the pneumococci and their absorption power were altered. Serum from rabbits immunized with immune-serum-treated pneumococci contained agglutinins for the homologous strain as well as for the heterologous immune-serum-treated strain, but no antibodies for pneumococci grown in normal serum. On the other hand, serum from rabbits immunized with pneumococci grown in normal serum agglutinated not only the homologous normal strain but reacted also, though less strongly, with immune-serum-treated pneumococci from heterologous as well as homologous strains.

By absorption of normal antipneumococcus serum with homologous immune-serum-treated pneumococci it was possible to

remove all antibodies for homologous as well as heterologous immune-serum-treated pneumococci, but not antibody for homologous normal-serum-treated pneumococci.

Stryker further found that absorption of normal antipneumococcal serum with normal-serum-treated homologous pneumococci removed agglutinins not only for these pneumococci but also for all immune-serum-treated strains. Stryker did not perform the reverse experiment: employment of homologous strains and normal-serum-treated pneumococci for absorption of serum from rabbits immunized with immune-serum-treated pneumococci. Finally, she showed that the pneumococci altered by treatment with immune serum could revert to the original type through animal passages, and that the number of passages depended on the number of serum treatments.

In 1921 *Arkwright* (5) described two kinds of colonies of dysentery, typhoid, paratyphoid B and enteritis bacilli obtained respectively from old cultures and from cultures on media containing immune serum, and he designated the colonies as S (smooth) and R (rough) because of their difference in appearance. *Griffith* (37) (1923) applied the S and R terms of *Arkwright* to the two forms of colonies which appeared when pneumococci were grown on media containing homologous immune serum. *Griffith* describes the S and R pneumococci as follows:

The S colonies are circular, dome-shaped, with smooth, shiny surface; when autolytic changes take place they become flattened and finally shaped like a button or checker. On growth in broth, where the growth is diffuse, a specific soluble substance is formed. On immunization of rabbits with such colonies a type-specific antibody is formed. Pneumococci from S colonies are agglutinated specifically by serum of the homologous type and they are virulent for animals.

The R colonies are somewhat smaller than the S colonies, and their surface is rough. But this morphological difference between S and R colonies is not constant, as R colonies may look exactly like S colonies. In serum broth the culture forms a sediment, and here the pneumococci have a tendency to form long chains. The R colonies are more adherent to the blood agar plate than the S colonies, and they are also more coherent, breaking

into pieces on being touched or sticking as a whole to the point of the needle at their removal. When grown in broth the R pneumococci form no specific soluble substance. On immunization of rabbits the R pneumococci induce merely a formation of species-specific antibodies, not type-specific. They agglutinate unspecifically, being agglutinated also by heterologous sera. R pneumococci are avirulent for animals and as a rule they can revert to the homologous S pneumococci through animal passages or repeated cultivation in blood broth. The reversion from R to S pneumococci depends partly on the number of passages in immune serum, partly on the activity of the immune serum employed, *i. e.,* the more transfers and the higher the titer of the serum, the more difficult will it be to get the R form reverted to homologous S pneumococci.

Concentrated serum was not necessary for dissociation of the pneumococci. Homologous serum diluted 1 : 256 produced a partial change of the pneumococci from the S form to the R form.

Anti-S serum agglutinated both S and R pneumococci, whereas anti-R serum gave only strong agglutination of R pneumococci, and a weak reaction with S pneumococci or none at all.

S pneumococci absorbed all antibody for R pneumococci as well as S from the homologous immune serum, whereas R pneumococci removed only the antibody for R from anti-S serum. The R pneumococci absorbed all antibody from anti-R serum, whereas the S pneumococci did not remove R antibody from anti-R serum but merely lowered the titer by one or two tubes. Anti-S serum protected the animals against S cultures, whereas anti-R serum gave no protection against S pneumococci.

Like *Stryker,* also *Blake & Trask* (9) (1923) reported such changes in the pneumococci on growth in homologous immune serum. These authors were of the opinion that the changes did not take place all at once in all the pneumococci, and that individual differences asserted themselves in this respect.

Amoss (3) (1925) examined one-cell cultures of Type 1 grown in 11 % Type 1 antiserum, bile broth or slightly acid broth and found the same as observed by *Stryker* and *Griffith:* that the one-cell culture dissociated into avirulent and virulent strains.

But *Amoss* did not succeed in getting the avirulent strains to revert to the virulent Type 1 strain. In the serum of rabbits immunized with the avirulent strain, agglutinins were formed but not protective antibodies against the original type. On cultivation of a single cell in heterologous or normal serum, no avirulent dissociants were formed.

Reimann (75) (1925) obtained R colonies by cultivating pneumococci in serum broth containing 1 % or 10 % homologous immune serum or bile in increasing concentrations, beginning with a concentration of 1 : 400 and ending with a concentration of 75 % bile, besides by repeated cultivation in normal serum or on blood agar plate. The resulting variants showed a lowered virulence for mouse and loss of their type specificity. The degree of the modification of the original culture was directly proportional to the relative number of pneumococci undergoing the variation, as the degree of virulence was dependent on the relative proportion between S and R pneumococci. If the variation manifested itself in all the pneumococci, the change was irreversible, and R pneumococci could not be transformed to S pneumococci by passage through mice.

In 1926 *Reimann* (76) reported some additional studies on the variation of pneumococci and found that immune serum produced by means of R pneumococci gave the same reactions as sera produced by injection of pneumococcal protein or autolytic products of pneumococci regardless of the pneumococcus type employed, *i. e.*, reactions with species-specific antigens, not with type-specific. *Reimann* further found that R pneumococci, regardless of their type origin, were serologically identical, possessing an antigen identical with pneumococcal protein; but he performed no cross-absorption of the R sera with different R cultures.

Jacobson & Falk (45) (1925-1926) found R pneumococci obtained from Type 1 by growth in homologous immune serum were unstable even after 23 mouse passages.

Levinthal (56) (1926), who obtained reversion of R pneumococci to S of Type 1 by means of one-cell cultures in ordinary serum broth incubated at 25° and following mouse passages,

thought that all R pneumococci under certain conditions would be able to recover their original type specificity and revert to their original form.

In a more comprehensive work on microbic dissociation *Hadley* (39) (1927) established that the dissociation of S pneumococci by growth in immune serum was dependent on three things: 1) the titer of the immune serum; 2) the concentration of the immune serum; and 3) the length of the time in which the bacteria were in contact with the immune serum. If the titer was too low, the serum too diluted and the contact between bacteria and immune serum not sufficiently protracted, no variants would arise.

In 1927, *Paul* (72) gave a description of the appearance of the R and S pneumococcus colonies under varying conditions. He investigated the influence of the following four factors on R and S pneumococci: 1) the size of the inoculum; 2) its age; 3) reduction in the blood content of the culture medium; and 4) the hydrogen ion concentration of the culture medium.

Dawson & Avery (24) (1927) transformed 7 out of 8 R pneumococci originating from Types 1, 2 and 3 to the homologous S type by means of mouse passages. The only exception was a Type 1 R pneumococcus, which had been cultivated for a long time and had thus lost the capacity for reversion. These authors further succeeded in transforming 6 R strains from Types 2 and 3 to the homologous S types by cultivation in media containing anti-R serum. In vitro, R 1 could not be made to revert. After the reversion these bacteria had acquired all the properties of the original S strains.

In two comprehensive papers of 1928 and 1930, *Dawson* (19-20) has reported his findings in studies on R pneumococci and the conditions for their reversion to the homologous S types. He succeeded in obtaining R pneumococci by cultivation in immune serum in concentrations of 1 : 1, 1 :2, and so on to 1 : 256; the optimal concentration was 10-25 %. He emphasizes that the morphology of the colonies alone could not be considered a decisive criterion of dissociation of the pneumococci, as the dissociation could be established only by specific agglutination and virulence tests.

Dawson worked with one-cell cultures of R pneumococci and he succeeded nearly always in getting R pneumococci to revert to S pneumococci; and in every instance of such reversion the R type was transformed to the same specific type as that from which it originated, and the transformation was always accompanied by the recovery of all the properties characteristic of the S type—as far as the virulence was concerned, too. The reversion to the homologous type was easier for R 2 and R 3 than for R 1.

In vitro the reversion of R pneumococci to S took place by cultivation in 10 % anti-R serum—but not if heat-killed vaccines of R pneumococci or other bacteria were added to the R culture at the same time.

In vivo the reversion succeeded when white mice were given subcutaneous injections of large amounts of a living R culture of small amounts of the R culture together with large amounts of a heat-killed homologous S culture. But the reversion failed if a heat-killed R vaccine or vaccine of other bacteria was used instead of the S vaccine.

Type 2 and 3 S vaccines were equally effective in inducing reversion, whether they had been heated to 60° for 15 min. or to 100° for 15 min. Type 1 S vaccine was effective in this respect when it had been heated to 60° for 15 min., not if heated to 100° for 15 min.

Kimura, Sukneff & Meyer (50) (1929) verified the observations reported by *Stryker, Griffith, Amoss* and *Reimann* by obtaining degraded pneumococci on cultivation in 10 % immune serum, but they did not find that the various R pneumococci were perfectly identical serologically—as was claimed by *Reimann.*

Klumpen (52) (1932) published a comprehensive systematic investigation into the formation of pneumococcal variants. He found that the most reliable method for the production of variants was to cultivate pneumococci in immune serum—10 %, 20 % or 33 %—and, like *Neufeld & Levinthal* (68), to cultivate in broth with addition of a piece of an organ (spleen, kidney or liver) from rabbits or guinea-pigs. These organs had to be boiled and stored in icebox for 8 days before use. All other ex-

periments (with bile, optochin or yeast, or with cultivation on solid media for a considerable length of time) resulted in dissociation but exceptionally.

Blake & Trask (10) (1933) demonstrated that the conversion from S to R pneumococci is not a simple change, and that the process of dissociation is associated with the appearance and disappearance of intermediate variants. Thus, on cultivation in 1.25 %, 5 % and 20 % homologous immune serum—of which the 5 % concentration proved the most suitable—the authors found 5 different variants between S 1 and R 1, and designated them as a, b, c, d and e. The intermediate forms showed distinct and progressive changes in their agglutination reactions in homologous and heterologous immune serum, besides a progressive loss of virulence for white mice. Strain a was more like S pneumococci, e more like R pneumococci. The authors found only one intermediate form between S 2 and R 2, and none between S 3 and R 3.

In 1934 *Dawson* (22, 23) reported some additional studies on the dissociation of pneumococci, with particular mention of a mucoid form that differed from S and R pneumococci, and which he called M. He showed that the change from the typical virulent S form to the degraded variants was no simple conversion from S to R, and that the dissociative process consisted in different phases, among which 3 distinct steps were represented by noticeable differences in the appearance and morphology of the colonies, symbolized by the letters: M, S and R.

Like *Blake & Trask*, also *Paul* (73, 74) (1934) obtained some intermediate forms. He was able to produce dissociants both in vitro and in vivo—in vitro by cultivation in 10 % homologous immune serum, in bile in increasing concentration, in optochin, and by letting the pneumococci grow on blood agar plate at room temperature.

Cotoni & Pochon (17) grew pneumococci in 10 % immune serum, and they also obtained intermediate forms besides R pneumococci.

Istrati, Dimitriu & Mesrobeanu (44) (1937) succeeded in getting R 1 to revert after respectively 3, 13, 34 and 48 passages in homologous immune serum by injecting large amounts of R 1

intraperitoneally together with homologous S vaccine, killed by heating to 60° for one hour. R 1 was formed by cultivation of S 1 in 10 % homologous immune serum—with the exception of the R pneumococcus formed after 48 transfers; this S 1 was cultivated only in 2.5 % homologous immune serum. One R 1 pneumococcus formed after 50 transfers failed to revert to the homologous S strain on intraperitoneal injection.

3. Transformation of Pneumococci.

In 1928 *Griffith* (38) published a comprehensive work on pneumococcus types in which he mentioned some studies on the dissociation of pneumococci, methods for reversion of the attenuated pneumococci to their virulent homologous S types, and transformation of pneumococcus types.

Griffith produced R pneumococci by growing the corresponding S pneumococci in homologous immune serum and showed that the R change is not equally advanced in all the dissociants. Some R strains formed a trace of soluble substance in the peritoneal cavity of mice; these strains readily reverted to S pneumococci and were able to induce active immunity. Other R forms contained no S antigen; they were unable to induce active immunity, and their reversion to the homologous S type was difficult. The stronger the immune serum used, the more permanent and complete was the change to the R form.

Griffith succeeded in making R pneumococci revert to the homologous S type either by subcutaneous injection of large amounts of R culture into white mice (the sediment from centrifuging of 50-100 cc. of a serum broth culture) or, preferably, by subcutaneous injection into white mice of small amounts of R culture together with large amounts of heat-killed S culture of the same type as the R bacteria. Sometimes the reversion was accomplished also by employment of a heterologous S type instead of the homologous. The S pneumococci were grown in glucose ox-serum broth. When the growth was good, the cultures were centrifuged, and the sediment was transferred to small sterile glass tubes, which were sealed and immersed in

water-bath of the desired temperature, which was watched closely and kept constant. Thus Type 1 antigen was found to lose the power of causing reversion of R to S when it was heated to 80°, whereas Type 2 antigen remained effective in this respect even after steaming at 100°.

More surprising than the reversion of the R form to the homologous S form was the transformation of type—from one specific type to another specific type—by subcutaneous injection of R pneumococci of one type together with heat-killed S pneumococci of another type.

The R form was derived from the S form by cultivation in homologous immune serum, usually 10 %. Blood broth cultures were used for these experiments. The S pneumococci were grown in glucose ox-serum broth. When the growth was good after incubation for a few hours, the culture was centrifuged, and the sediment was transferred to small glass tubes, which were sealed and heated by immersion in water of the temperature wanted.

The R culture was injected—in a dose of 0.2-0.25 cc.—subcutaneously over the right inguen in mice together with the sediment of the heat-killed S culture in a dose of up to 100 cc. of culture. When the mouse died, autopsy was performed as follows:

The mouse was pinned out, and the abdominal skin was seared with a hot iron but not exactly over the site of injection. Through the seared area an incision was made with a sterile knife, and the skin was reflected back with a pair of forceps until the site of injection was exposed, and then it was pinned down. A fresh pair of sterile instruments was used to scrape the subcutaneous tissue, and usually the lymph gland in the groin was removed at the same time. The material was placed in a small tube with a few drops of broth, in which it was rubbed up. A loopful of the fluid was spread on a plate, and to the remainder a little blood broth was added. If the next day, after incubation, the fluid culture on microscopic examination of smears showed any diplococci, it was plated too. Cultures were made also from the heart's blood on plate and in broth. The new-formed type was isolated from the site of the injection and as a rule from the heart's blood too.

In this way *Griffith* succeeded in transforming R 2 to S 1 and R 1 to S 2. Furthermore, S 3 was formed both of R 1 and R 2, though apparently more readily from R 2. The new-formed S 3 pneumococci were relatively low-virulent; often they were found only subcutaneously, and they produced no septicemia. As *Griffith* had no Type 3 serum, the diagnosis was made on the appearance of the colonies and on the failure of the culture to agglutinate with Type 1 and 2 sera.

The S 1 and S 2 antigens were found to be rendered unserviceable for transformation of type by heating to the same temperatures at which they lost their power to cause reversion of R to S, and Type 1 antigen proved more sensitive than Type 2 antigen also in this respect.

Griffith also succeeded in producing transformation of R pneumococci derived from an avirulent Group 4 strain to virulent strains of Types 1 and 2.

In contrast to all the above-mentioned experiments, *Griffith* was unable to obtain any transformation of type when instead of using a heat-killed S culture he employed a heat-killed R culture together with small doses of living R culture.

Nor did attempts at transformation of type in vitro turn out successfully. In test tubes containing ox serum he mixed an R 2 culture with heat-killed S pneumococci, corresponding to the sediment of 100 cc. of culture. After incubation for 24 hours a loopful of the mixed cultures in the tubes was spread on blood agar plates, and the tubes were centrifuged; the supernatant serum was pipetted off and sediment stirred up again in sterile ox serum, and then incubated. This procedure was repeated 8 times, but all the plates showed growth only of R pneumococci.

Neufeld & Levinthal (68) (1928) confirmed the findings of *Griffith* concerning transformation of type, but they employed a somewhat different method for the production of R pneumococci, growing the virulent type-specific strains in broth containing sterile animal organs (liver, spleen or kidney of rabbits). The attenuated pneumococci were injected subcutaneously in the left hypochondrium together with heat-killed S pneumococci. The S pneumococci were grown in 2 % dextrose broth for 5

hours, and the culture was then centrifuged and heated in sealed tubes to 60° for 20 or 40 min. In this way the author succeeded in transforming R 1 to the homologous S type and to the heterologous S 2 type.

Reimann (77) (1929), for whom the transformation of type also turned out successfully, though not as easily as for *Griffith*, employed in the transformation experiments stable R pneumococci, *i. e.*, S pneumococci grown in several subcultures in 10 % immune serum. These stable R pneumococci could not be reverted to the homologous type by the usual methods. *Reimann* cultivated S pneumococci of Types 1, 2 and 3 in 1 % dextrose broth and heated the sediment to 60° for 15 or 40 min. And by means of these vaccines he was able to transform R 1 to S 2 and S 3, and R 2 to S 1 and S 3.

Dawson (21) (1930) established and elaborated *Griffith*'s observation. He let S pneumococci grow in ordinary broth at 37° for 10 hours, as longer growth and the resulting autolysis had an injurious effect on the vaccine. *Dawson* found that S vaccine of Types 1, 2 and 3, heated for 15 min. at 60-80°, was able to transform R pneumococci derived from heterologous S forms to the type of the vaccine employed. If, on the other hand, the vaccine was heated to 80-100° it was not effective in this respect while S vaccine of all three types heated to 80-100° was able to cause reversion of R 2 and R 3 to their homologous S types, but not R 1 to the original S type. S vaccine of Type 1 heated to 60° for up to 2 hours was able to transform heterologous R to Type 1. Large amounts of heat-killed pneumococci were required to induce the transformation. S pneumococci corresponding to 100, 50 and 25 cc. of culture were able to induce transformation, whereas S pneumococci from 10 cc. of culture were ineffective in this respect.

Dawson also worked with single-cell cultures of R pneumococci and was able by employment of the respective R forms successively to transform Type 2 into Type 3, and this into Type 1 and this again into types of Group 4.

Dawson further injected autolysates of S cultures subcutaneously into white mice together with a living R culture and showed that the autolysates were unable to cause reversion of

R to the homologous type or to the type from which the autolysate was derived. For autolysis of the S pneumococci the S culture was centrifuged and, before heating, incubated at 37° for 48 hours. On the other hand, heat-killed S pneumococci were effective in bringing about reversion even after storage in ice-box for 3 weeks.

S cultures killed in some way other than by heating—for instance, by means of formalin, iodine, chloroform, tricresol, alcohol, or acetone—were of no use for the transformation of type.

Dawson tried intraperitoneal injection of small amounts of R forms together with large amounts of heated heterologous S cultures and found that the transformation of type was effectuated far more easily with the subcutaneous method than with the peritoneal, as with the latter method he succeeded only once in transforming R 2 to Type 3 S.

Inoculated subcutaneously with R and S pneumococci, the mice died at the earliest 18 hours after the injection of S pneumococci of the same type as the vaccine, and in this length of time the new-formed S pneumococci had developed in a number sufficient to kill the animal; and probably the time required for the reversion at the site of injection would be much shorter.

Dawson further gave a series of mice a subcutaneous injection of S vaccine and 2, 4, 8, 12 and 24 hours later an injection of living R pneumococci at the same site. No reversion took place in any animal inoculated with R pneumococci later than 8 hours after the injection of the S vaccine.

When R and S pneumococci were injected into mice in different places—for instance, subcutaneous injection of S pneumococci in one groin and R pneumococci in the other—transformation of type took place only a few times, and in such positive experiments *Dawson* could not exclude the possibility that the living R pneumococci might have been carried over to the heat-killed S pneumococci and that this brought about the transformation.

Dawson also attempted direct transformation of S pneumococci of a specific type to S pneumococci of another specific type by injecting small amounts of living S culture (in dilutions 10^{-7}, 10^{-8} and 10^{-9}) together with large amounts of a hetero-

logous S vaccine, but all the mice died of pneumococcus septicemia of the same type as the living culture.

Finally *Dawson* tried to obtain transformation of type in vitro. Large amounts of S 1 vaccine were injected intraperitoneally in 5 white mice, and the animals were killed 2, 4, 8 and 12 hours later. The peritoneal cavity was flushed with ordinary broth, which then was poured into test-tubes and mixed with R 2 cultures; but in no instance was a transformation of type obtained in vitro.

Dawson thought that if the R pneumococci were reduced to the final stage of »degradation« they might be transformed after *Griffith's* method into heterologous S pneumococci. If, on the other hand, the R pneumococci were degraded but partially, they would revert to the homologous type. If the vaccine was heated to a temperature between 60 and 80° the R pneumococci would be transformed to the type of the vaccine. If the vaccine was heated to more than 80°, the R pneumococci would revert to the homologous type.

In 1931 *Dawson & Sia* (25, 26) gave as the first a technique by which it was possible to obtain transformation of pneumococci in vitro. The requirements of this transformation were: minimal amounts of R culture, addition of heated activating S pneumococci, incubation for a longer period than usual, and addition of small amounts of 10 % anti-R serum and blood broth.

Dawson & Sia's first experiment in vitro consisted in intraperitoneal injection into mice of S 3 vaccine, corresponding to 100 cc. of culture. The animals were killed after 6 hours, and the peritoneum washed thoroughly with sterile saline, which then was transferred to test-tubes. One drop of a 10^{-6} solution of an R 2 culture was then added, and the tubes were incubated for 24 hours. In one instance typical Type 3 colonies appeared on the plates inoculated from the tubes. In the next experiment *Dawson & Sia* used 1 cc. of ordinary broth instead of saline for the lavage of the peritoneal cavity, and the broth was then transferred to small sterile tubes, and 0.1 cc. of anti-R serum was added to half of the tubes. All the tubes were inoculated with one drop of R 2 culture in dilution 10^{-6}, and incubated at 37° for 4 days, blood agar plates being inoculated from the tubes

at intervals of 24 hours. In this way, *Dawson & Sia* obtained typical S 3 colonies from all the tubes to which anti-R serum was added, whereas Type 3 pneumococci were obtained from the other tubes only in one instance.

Experiments were then performed with the filtrate of the broth used for the peritoneal lavage. The broth was filtered through a Berkefeld filter; otherwise the experiment was carried out exactly as before. No transformation of type took place, with or without addition of anti-R serum.

Dawson & Sia then tried whether transformation of type might be accomplished entirely in vitro. 0.5 cc. of Type 3 S vaccine, corresponding to 100 cc. of culture, was inoculated with 1 drop of R 2 culture in dilution 10^{-6}, whereafter 0.5 cc. of blood broth was added to all the tubes and, in addition, 0.1 cc of anti-R serum to half of the tubes. All the tubes were incubated at 37°, and blood agar plates were inoculated from them every day. Typical Type 3 pneumococci were formed in all the tubes, with and without addition of anti-R serum. *Dawson & Sia* further estimated how large amounts of R culture had to be added to heterologous S vaccine in order to obtain transformation of type. The experimental technique was the same as before, but the addition of R culture varied, one drop of R culture being added to the tubes in the following dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. In this way, the authors found, transformation took place but rarely or almost never when the concentration of the R culture was greater than 10^{-2}, and nearly always with addition of small amounts of R pneumococci. They further estimated that the smallest amount of S vaccine required for transformation of type in vitro corresponded to 0.1 cc. of the original culture, smaller amounts being quite ineffective.

Dawson & Sia also investigated which culture media were most suitable for the process of transformation and found the transformation to take place in ordinary broth with addition of normal rabbit serum or anti-R serum, in blood broth alone, and in blood broth with addition of normal rabbit serum or anti-R serum, whereas no transformation took place on employment of ordinary broth alone. The most suitable medium was blood broth with an addition of anti-R serum.

The temperature to which the S vaccine could stand heating without jeopardizing the transformation of type in vitro, was found to be the same as for the experiments in vivo. The authors thus found that S vaccine heated for 15 minutes to 60-80° was effective in the induction of transformation of type, whereas vaccine heated for 15 min. at 90° or 100° proved ineffective. S vaccine heated 3 times to 60° for 15 min., 2 times to 70° for 15 min., or once to 80° for 15 min. proved still effective in causing transformation of type. On the other hand, the vaccine was unserviceable when it was heated 3 times to 70° for 15 min., or twice to 80° for 15 min. Vaccine S 3 heated to 60° for 4 hours was still effective in bringing about transformation of type but less effective than the vaccine heated to the same temperature but only for 1 or 2 hours.

With this method *Dawson & Sia* succeeded in transforming R 2 to S 3 and S 1 in vitro.

The same year, 1931, *Sia & Dawson* (80) reported some additional studies on transformation of type in vitro. They found that R pneumococci possessing a slight degree of stability were most suitable for the transformation, and that the experiment would turn out negative if the vaccine itself was replaced with a supernatant fluid resulting from hard centrifuging of the vaccine, with the filtrate of heat-killed S pneumococci, with an actively growing S culture, or with the purified soluble substance.

The authors further performed some experiments with cell-free extracts of S pneumococci prepared by repeated freezing and thawing of the vaccine, in which they found that S vaccine killed by heating to 60° for 30 min. and then submitted 23 times to freezing and thawing still remained effective, whereas the vaccine was incapable of transformation if it had been frozen and thawed first and then heated to 60° for 30 min. The filtrate from heated, frozen and thawed S cultures and filtrate from the same material after primary freezing and thawing and subsequent heating proved ineffective. The freezing and thawing had taken place under access of the air, but even when this treatment was carried out under anaerobic conditions the result was the same. The suspension of S pneumococci which first was heated to 60° for 30 min., and then frozen and thawed 20 times

under anaerobic conditions still proved effective in the induction of transformation of type. On the other hand, the supernatant fluid of the S vaccine was ineffective when the vaccine had been heated to 60° for 30 min., then frozen, thawed and centrifuged, and also when it was first frozen and thawed anaerobically and then heated to 60° for 30 min., and centrifuged.

Finally, *Sia & Dawson* showed that vaccine which had been submitted to the action of bacterial enzymes (*e. g.*, in old cultures) was not able to induce transformation and thus cultures over 48 hours were unserviceable.

Dawson & Warbasse (27) (1931) have described the transformation in vitro of a specific S pneumococcus type to another specific type apparently without the development of any intermediate form. A single-cell culture of Type 2 S was employed for these experiments. A drop of a 10^{-6} dilution of the culture was transferred to the culture medium which contained homologous immune serum together with large amounts of Type 3 vaccine in which S 3 had been killed by heating. The culture was incubated at 37° and after 48 hours it presented several Type 3 colonies besides Type 2 colonies, but no R pneumococci. On addition of heterologous immune serum Type 1 instead of the homologous Type 2 serum, transformation of Type 2 to Type 3 was likewise obtained without formation of R forms.

Even though the conditions in these experiments were unfavorable for the development of R forms, in particular on addition of heterologous serum, *Dawson & Warbasse* still found it more likely that the S pneumococci passed through this intermediate stage in the process of transformation.

Baurhenn (8) (1932) was the first to report reversion of R pneumococci to the homologous and heterologous S pneumococci within Group X. He worked with R 1, R 2, R 3, R 6, R 8, R 10, R 11 and R 24, and with several strains within each type formed in different ways, either by growth in homologous immune serum, in broth with organs of guinea-pig or rabbit ad modum *Neufeld & Levinthal* (68), and in bile broth, or by growth on blood agar for a considerable length of time. As culture medium for the R pneumococci he used 10 % ascites broth

or slightly alkaline fructose broth. The S pneumococci were grown in 2 % fructose broth with pH = 7.6. The vaccine was prepared by heating the S pneumococci to 60° for 15-40 min. or, more frequently, to 56° for 75 min.

In numerous experiments *Baurhenn* obtained transformation of R 2 to S 8, R 6 to S 11 and S 29, and R 10 to S 29, but no transformation of R 1 to S 2 or S 8, R 2 to S 1, S 3 or S 19, R 3 to S 1, S 2 or S 11, R 6 to S 2 or S 3, R 8 to S 2, S 3 or S 11, R 10 to S 1, S 8 or S 11, R 11 to S 1 or S 8, and R 24 to S 1.

Baurhenn thought that readily degraded R strains reverted to their homologous S strains, and moderately degraded R pneumococci to the heterologous S pneumococci, whereas stable R pneumococci were difficult to transform to the homologous or heterologous S pneumococci.

In 1932 *Alloway* (1) succeeded in extracting the transforming factor from the pneumococcus cell.

Alloway grew the S pneumococci in beef broth with pH = 7.8 and 0,01 % dextrose. Immediately before its inoculation, this culture medium was boiled for 10 min. and then cooled quickly to a temperature suitable for the growth of pneumococci. The cultures grew under a cover of vaseline or liquid paraffin for 10-12 hours, depending on the density of the growth.

After centrifuging of a 5-liter broth culture the pneumococci were suspended in about 40 cc. of distilled water which then was transferred to a thick pyrex tube and immediately covered with a layer of liquid paraffin. This concentrated suspension was then frozen in broth repeatedly until the bacteria were broken into pieces—as a rule, 7-8 freezings were required. The freezing of the mixture was carried out with dry ice in alcohol. After destruction of the cells the mixture was immediately placed in water-bath and heated to 60° for 30 min. and then centrifuged at a high rate of revolution for 2½ hours. The supernatant fluid was diluted with sterile water to a volume of 500 cc., made alkaline by addition of 1.0 cc. n sodium hydroxide, and filtered through a Berkefeld N filter. The filtrate was neutralized partially by addition of 0.7 cc. n hydrochloric acid. The solution was then concentrated by vacuum distillation at a low temperature to 50 cc., *i. e.*, $^1/_{100}$ of the volume of the original

broth culture, and finally the extract was heated in water-bath to 60° for 10 min. in order to ensure sterility.

The anti-R serum employed by *Alloway* was produced by intravenous injection into rabbits of a suspension of heat-killed R pneumococci. Later he used normal swine serum instead of anti-R serum, as he found it just as serviceable, and it was easier to obtain.

With filtered extract prepared in the above-mentioned manner from S 1 and S 3, *Alloway* was able in vitro to transform R 2 to S pneumococci of the specific type from which the extract was obtained, but he did not succeed in transforming R 1 or R 3 to any heterologous S form in vitro. These experiments were carried out as follows:

One drop af an R culture prepared by dilution of 0.1 cc. of an 8-hour culture in 5 cc. of sterile broth was added to a test-tube containing 1.5 cc. broth, 1.0 cc. filtered extract and 0.3 cc. of swine serum. The mixture was at once covered by a thick layer of sterile liquid paraffin, and incubated at 37°. Subcultures were made every 24 hours, one drop of the culture being transferred to another series of tubes containing the same amount of broth, extract and swine serum. In no experiment was the result considered negative before at least 5 subcultures had been made. Blood plates were inoculated simultaneously with the subcultures, and the resulting colonies were examined.

Alloway also carried out some experiments in vitro with purified polysaccharide from the pneumococcal capsule but was unable to obtain any transformation of type with purified specific substance.

In the following year, 1933, *Alloway* (2) reported additional studies on pneumococcus extract employed for induction of transformation of type in vitro. This pneumococcus extract was prepared as follows:

The S pneumococci were grown for 8-10 hours in beef broth with pH = 7.8 and 0.01 % dextrose. The bacteria from 5 liters of culture were separated by centrifuging and transferred into 50 cc. of sterile distilled water. To this suspension was added 3.5 cc. of a sterile 10 % solution of sodium desoxycholate, and this mixture was placed for 10 min. in ice-bath, then heated

slowly to 60° in water-bath. In this way the pneumococci were dissolved rapidly and formed a thick, fairly tenacious gell, which was kept in water-bath at 60° for 15 min.—in order to kill surviving bacteria and inhibit or destroy the autolytic enzymes liberated by the solution of the cells. To this bacterial solution was slowly added 500 cc. of absolute alcohol which had been chilled in ice-bath. This produced a thick solid precipitate, which settled slowly when the mixture was left standing at room temperature; the sodium desoxycholate, being soluble in alcohol, remained in the supernatant fluid. The precipitate was separated by centrifuging for 30 min., and the supernatant fluid was poured off. For removal of the last trace of bile salts, the precipitate was washed with alcohol and then dried in vacuum or by means of a cold dry current of air for 12-14 hours. The dried material was now extracted in 100 cc. of sterile 0.85 % sodium chloride solution, made slightly alkaline by addition of 0.5 cc. of $^1/_{10}$ n sodium hydroxide and transferred to small glass tubes which were sealed and heated to 60° for 15 min. in water-bath. The preparation was then centrifuged for 30 min., and the insoluble remnant, which contained only small amounts of active material, was removed.

Instead of anti-R serum or swine serum as employed by *Alloway* in his preceding experiments, he now used ascitic fluid or pleural effusion, which proved just as serviceable as anti-R serum. The pleural exudate was obtained from a patient with heart lesion and general anasarca.

The experiments were carried out with almost the same technique as the preceding. One drop of a 10^{-4} solution of an 8-hour R culture was added to a series of tubes, which contained 1.5 cc. of broth, 0.5 cc. of pleural exudate and 0.05-0.1 cc. of the specific pneumococcus extract. The cultures were incubated under anaerobic conditions at 37° for 24 hours. Every 24 hours 1 drop of the culture was transferred to a new series of tubes, and a blood agar plate was inoculated at the same time. No experiment was reckoned as negative till 5 series of subcultures had been performed without resulting in transformation of type.

Pneumococcus extract prepared by means of sodium desoxycholate proved more effective in the induction of transformation

of type than extracts obtained from frozen and thawed bacteria. In high dilution they were able to induce transformation, but not in strong concentration. By means of this extract R 2 was transformed to S 3 in vitro.

By additional purification of the material *Alloway* tried to isolate the activating substance; and for this purification, among other means, he used powderized charcoal.

100 cc. of extract produced in the above-mentioned manner was diluted with 100 cc. of sterile 0.85 % sodium chloride solution, and to this was added 8 g. of sterile powderized charcoal The mixture was shaken for 5 min., and then centrifuged at the highest rate of revolution for 30 min. The supernatant fluid was filtered through sterile filter paper and Berkefeld filter. *Alloway* found extracts purified in this way to be even more effective than the crude extracts in inducing transformation of type. With this extract R 1 was transformed into S 2, R 2 to S 1 and S 3, and R 3 to S 2, but not R 3 to S 1.

Additional purification of the charcoal-absorbed extracts was obtained by *Alloway* by precipitation in acetone or alcohol. The filtered extract was precipitated at a low temperature with 10 volumes of acetone, and after standing for one hour it was centrifuged strongly for 30 min.; the supernatant acetone was removed; and, after drying in vacuum, the precipitate was transferred to the original volume of distilled water. The insoluble rest was removed after centrifuging, and the supernatant fluid contained the active factor without any loss worth mention. Instead of acetone, alcohol was employed with the same success. By means of this new extract *Alloway* obtained transformation of R 2 to S 3.

The transforming principle which he thus obtained in extracts was found to be more heat-resistant than the vaccine prepared from whole pneumococcus cells. Thus, the purified preparations were sometimes found to be active even after heating in water-bath to 90° for 10 min., although they showed a progressive decrease in the capacity for transformation on heating to over 80°. Boiling, on the other hand, destroyed the activity of the purified extract in every instance.

Alloway further showed that the purified extract did not

lose the faculty of inducing transformation even after filtration through Berkefeld N and W filter if only the extract was alkaline. If it gave an acid reaction, the filtration resulted in complete loss of this activity.

Harris (41) (1938) described transformation with *Dawson & Warbasse's* technique of a high-virulent type to a low-virulent and, conversely, of a low-virulent type to a high-virulent; and he showed that the virulence of the new-formed pneumococcus corresponded to that of the type from which the capsule originated. Thus, a Type 2 pneumococcus transformed to Type 14 would have the same virulence as Type 14, *i. e.*, a low virulence; conversely, a Type 14 pneumococcus transformed to Type 2 would have the same virulence as Type 2, *i. e.* a high virulence.

4. *Sensitivity of Pneumococci to Heat.*

In 1923 *Lange* (54) showed that pneumococci could stand heating to 52° for 15-90 min. In more extensive experiments, *Lange & Keschichian* (55) showed that pneumococci were killed by heating to 52° for 2½ hours but not within a shorter time; and they were able to stand heating to 55° for 15 min., but not for 30 min.

Paul (74) (1934) mentioned that pneumococci are killed by heating to 56° for 20 min., while *Christensen* (11-12) (1922-23) gave heating to 56° for 30 min.

Avery (6) (1915), *Stryker* (84) (1916) and *Baurhenn* (8) (1932) asserted that pneumococci were killed by heating to 56° for 75 min.

Robinson (78) (1927), *Griffith* (38) (1928), *Reimann* (77) (1929), *Dawson* (20-21) (1930), *Dawson & Sia* (25-26) (1930-31), *Dawson & Warbasse* (27) (1931) and *Alloway* (1-2) (1932-33) claimed that heating to 60° for 15 min. was sure to kill pneumococci.

Yoshioka (91) (1922) and *Neufeld & Levinthal* (68) (1928) showed that the pneumococci were killed by heating to 60° for 20 min.

Gay, Atkins & Holden (36) (1931) investigated the influence of the temperature on R and S pneumococci in culture as well as

in dried form and found the R culture to be more heat-resistant than S. Thus R cultures could stand heating to 56° for 30 min., while S cultures were killed by heating to 56° for 15 min. Neither the R nor the S culture could stand heating to 60° for 15 min. When the pneumococci were dried in desiccator, both the R and the S forms could stand heating to 115° for 15 min. but were killed at 120° for 30 min.

Numerous tests for sterility in vivo and in vitro have been recorded by various investigators. The vaccine was inoculated on blood agar plates and in ox serum, blood broth, serum broth, blood-extract dextrose broth and ascites broth immediately after the heating and after standing in incubator at 37° for one or several days. The samples of vaccine to be tested were incubated for up to two weeks, and numerous blood plates were inoculated from the tubes. The experiments were carried out under anaerobic as well as aerobic conditions (*Dawson* (20-21)), but living pneumococci were never demonstrated at the temperatures mentioned by the investigators.

Tests for sterility were carried out in vivo by injecting the heated vaccine alone, subcutaneously over the groin in mice, in amounts corresponding to 130 cc. of culture or less. Most of the mice survived and were killed from 7 to 21 days after the injection. Cultures were made on blood plates and in broth from the site of injection and from the heart's blood. The inguinal gland and some of the subcutaneous tissue were divided finally and mixed with a little blood broth and spread on blood agar plates. All the samples of vaccine examined were sterile. Further, some of the last-mentioned mixture was injected intraperitoneally into mice, and all these mice survived.

A few mice which were given an injection of the vaccine alone died; but the presence of pneumococci could be demonstrated neither at the site of the injection nor in the heart's blood.

Dawson (20-21) also injected the vaccine intraperitoneally into mice, and none of these animals died of pneumococcus septicemia.

In one instance—a control mouse which had received an injection of vaccine corresponding to 70 cc. of Type 3 culture—

Griffith (38) found one smooth pneumococcus-like colony on the blood agar plate inoculated from the site of the injection. This colony was bile-soluble and virulent for mouse, did not agglutinate in Type 1 and 2 serum, but did not look watery as a normal Type 3. The broth culture from the site of injection showed no growth of diplococci after incubation for 24 hours, and the blood agar plate inoculated with the broth was sterile. *Griffith* thought that the pneumococcus colony in this case did not originate from the injected vaccine but from contamination of the plate, as there was only one colony on this plate, and all other sterility tests proved the vaccine to be sterile.

Neufeld & Levinthal (68) carried out sterility tests by subcutaneous injection of vaccine together with colon bacilli, and in no instance did they obtain growth of the vaccine pneumococci.

Dawson (20, 21) injected pneumococcal vaccine into mice together with living bacteria—*e. g.*, staphylococci, streptococci, influenza bacilli, and Friedländer's bacillus—and also vaccine alone into alcohol-intoxicated mice, but no living pneumococci were demonstrated in this way either.

On the other hand, *Haendel & Lange* (40) (1929) thought that pneumococci were able to survive heating to 60°. They investigated this quality of Types 1, 2 and 3 in serum broth cultures and in saline suspensions and asserted that pneumococci in protein-containing culture media and, occasionally, in saline suspension were able to stand heating to 60° for 30 min.—better in serum broth than in saline. The growth of these pneumococci was demonstrated by cultivation and by inoculation of mice. A few times, the authors claimed, they obtained growth also from serum broth cultures that were heated to 70° for 30 min., but the cultures were not immersed under the surface of the water-bath throughout this period. In one of their tables growth is recorded for pneumococci in serum broth heated to 60° for 90 min., but undoubtedly this is an error in the table, as it is not mentioned in the text.

In 1937-38 *Dunlop* (29) reported some studies on the sterility of vaccine prepared after the method given by *Dawson & Sia* (25, 26) and arrived at the result that the vaccine was not sterile. *Dunlop* thought that *Dawson & Sia* had failed to find living

pneumococci because they had used too small amounts of vaccine in their control tests for sterility, and because they had not incubated their control cultures long enough at 37°.

Dunlop examined vaccines of Types 2 and 3 by cultivation on 5 % blood agar plates, in 5 % blood broth and in 10 % serum broth. The blood agar contained 1.5 % agar; normal horse serum was employed for the serum broth. The pneumococci were grown in ordinary broth, and all the vaccine prepared from these cultures was used for examination for sterility; but *Dunlop* fails to mention how much vaccine he placed in each tube of serum broth or blood broth and on the blood agar plates. Nor does he mention to what degree and how long he had heated the vaccine. The sterility tubes were incubated at 37° for up to 3 weeks after their inoculation, but no growth of pneumococci was obtained after 8 days of incubation. As a rule, growth was demonstrated on the fourth or fifth day of incubation.

PART II

WRITER'S STUDIES

Chapter I.

TECHNIQUE

1. *Culture Media.*

The culture media employed in my experiments and their composition are as follows:

Broth:

Veal or beef	500 g.
Peptone (Riedel)	10 g.
Sodium chloride	3 g.
Secondary sodium phosphate ...	2 g.
Tap water	1000 g.

pH 7.3-7.4

Serum Broth:

Broth with addition of 5 % ox serum.
pH 7.3-7.4

Serum Broth with Glucose:

Serum broth with addition of 0.05 - 0.10 - 0.15 - 0.20 - 0.25 or 0.3 % glucose.
pH 7.3-7.4

Blood Broth:

Broth with addition of 5 % normal defibrinated horse blood.
pH 7.3-7.4

Horse Serum Broth:

Broth with addition of 10 % normal horse serum.
pH 7.4-7.5

Agar:

Broth with addition of 1.85 % agar.
pH 7.3-7.4

Blood Agar:

Agar with addition of 10 % sterile defibrinated horse blood.
pH 7.3-7.4

Dunlop's Blood Agar:

Agar, containing only 1.5 % agar, with addition of 5 % sterile defibrinated horse blood.
pH 7.3-7.4

Truche:

Peptone Chapoteaut	40 g.
Glucose Merck	5 g.
Sodium chloride	5 g.
Tap water	1000 g.

pH 7.8

Ox Heart Broth:

Ox heart	500 g.
Peptone (Orthana)	10 g.
Sodium chloride	2 g.
Secondary sodium phosphate	2 g.
Glucose	2 g.
Distilled water	1000 g.

pH 7.6

Martin Broth:

Swine stomach	300 g.
Concentr. hydrochloric acid	10 g.
Distilled water	1000 g.

Veal	500 g.
Distilled water	1000 g.
Glucose	3 g.

pH 8.1

Preparation: 18 swine stomachs (about 9 kg.) are cleaned, freed from fat and chopped. 300 g. of chopped swine stomach is added to 1 liter of distilled water and 10 cc. of concentrated hydrochloric acid. The mixture is placed in water-bath at 50-55° for about 15 hours, and then the temperature is raised to 80° and kept at this for 1 hour. The mixture is cooled and placed in ice-box (4°) for 24 hours. After removal of the fat, the swine stomach extract is filtered through flannel and measured.

Besides, 500 g. of chopped veal is steeped in 1 liter of water for 24 hours, whereafter the mixture is boiled for 15 min., and the meat is separated from the broth by pressing.

This broth is mixed with equal parts of the swine stomach extract and heated to 80°, alkalized to pH ca. 8.3, filtered through filter paper, autoclaved for 15 min. at 110°, filtered again through filter paper and measured; 1.5 % glucose is added, and the broth is autoclaved for 15 min. at 110°.

Trypsin Broth:

Horse meat	18135 g.
Pancreas	500 g.
Glacial acetic acid	600 g.
Baker's yeast	62 g.
Maltose	180 g.
Tap water	60000 g.

pH 8.0

Preparation: 18135 g. of chopped fat-free horse meat is mixed with 20 liters of tap water and heated by steam to 80° under vigorous stirring. When the temperature reaches 80°, the steam is shut off, and 40 liters of cold water is added, which lowers the temperature to about 50°. The reaction of the mixture is adjusted to pH = 8.0 by means of anhydrous sodium carbonate (about 150 g.). The trypsin emulsion (500 g. pancreas) is added in amounts of 150 cc. at intervals of half an hour (5 - 6 - 7 additions). During the digestion the temperature is kept at 50°, and pH at 8.0. After 3 hours, 600 cc. of glacial acetic acid is added, and the mixture is boiled strongly for 35 min., whereafter it is filtered through cloth and placed in ice-box (4°) for 12 hours. Then it is heated to 30°, and pH is adjusted to 7.0 with 5 n NaOH. 62 g. of baker's yeast, stirred up in a little broth, is added. The temperature is kept at 30° for 1 hour, pH is adjusted to 8.0 with 5 n NaOH, the mixture is heated to 100°, and 0.3 % maltose is added.

Wollstein's Blood Extract Broth:

Broth is mixed with equal parts of sterile defibrinated horse blood.

pH 7.4

(see *Kahlfeld-Wahlich:* Bakteriologische Nährboden Technik. 2' edition, Berlin 1922, p. 11.)

Thin Agar with Wollstein's Blood Extract Broth:

Broth with addition of 0.8 % water-agar (2.5 %) and 10 % blood extract broth.

pH 7.4

2. *Cultures.*

The experiments were carried out with capsulated pneumococcuc strains of Types 1, 2, 6, 8, 14, 20, 22 and 35—designated as S 1, S 2, etc.—and with the corresponding non-capsulated pneumococci designated as R 1, R 2, etc.

Type 1 was Neufeld's original Type 1. Type 2 designated as M 57, Type 6 designated as Fiorelli and Types 14, 20 and 22 originated from America. Types 8 (No. 12455) and 35 (No. 361/39) were isolated here in the State Serum Institute.

Both the capsulated and the non-capsulated pneumococcus strains were stored till their employment either in the form of dried serum broth cultures or in ice-box (4°) in »Truche« with addition of gelatine.

For the experiments, about 1 cc. of the Truche culture was transferred to blood broth and incubated at 37°, and then the S pneumococci were injected intraperitoneally on mice in order to increase their virulence. When the mouse died 12-24 hours after the injection, autopsy was performed, and cultures were made from the heart's blood in serum broth From this culture, subcultures were made in the ox heart broth or serum broth employed in these experiments. The R pneumococci were transferred directly from blood broth to serum broth. The dried cultures were mixed with blood broth and incubated. On the following day the broth culture was injected into mice, or subcultures were made in serum broth.

Besides pneumococcus cultures, also cultures of colon bacilli, streptococci and staphylococci were employed.

The colon bacillus cultures were 8-10-hour cultures in ordinary broth. The strains (colon 491 and colon Ali) originated from F. Kauffmann's collection of colon strains in the State Serum Institute. The broth was inoculated from a blood agar plate with the colon strain in pure culture.

The streptococcus and staphylococcus cultures were 8-10-hour cultures in serum broth. The streptococcus strain here employed was Streptococcus fæcalis 905 from Friedberg's collection of non-hemolytic streptococci in the State Serum Institute. The staphy-

lococcus strain originated from an ordinary plate contamination with Staphylococcus aureus.

3. *Tests for Virulence.*

Prior to each experiment, in most instances, the virulence of the capsulated pneumococci employed in the experiment was determined as follows: Various dilutions of the pneumococcus culture were made in 8 tubes after the following schema:

Tube	1:	9.5 cc.	broth	+	0.5 cc.	pn.	culture,	*i. e.*,	dilution		1:20
»	2:	9.0 cc.	»	+	1.0 cc.	from	tube	1,	»	»	1:200
»	3:	9.0 cc.	»	+	1.0 cc.	»	»	2	»	»	1:2000
»	4:	9.0 cc.	»	+	1.0 cc.	»	»	3	»	»	1:20000
»	5:	9.0 cc.	»	+	1.0 cc.	»	»	4	»	»	1:200000
»	6:	9.0 cc.	»	+	1.0 cc.	»	»	5	»	»	1:2000000
»	7:	9.0 cc.	»	+	1.0 cc.	»	»	6	»	»	1:20000000
»	8:	9.0 cc.	»	+	1.0 cc.	»	»	7	»	»	1:200000000

A 10 % blood agar plate was inoculated with 0.2 cc. of the dilution in tube 5 and incubated at 37° for 24 hours; then the pneumococcal colonies were counted. Two mice were inoculated intraperitoneally with 0.2 cc. from each of tubes 5, 6, 7 and 8. When the mice died, cultures were made from the heart's blood on 10 % blood agar plate and in serum broth, which after incubation at 37° were examined for colonies of capsulated pneumococci.

4. *Preparation of Vaccine and Specific Soluble Substance (SSS).*

The vaccine for the experiments and for immunization of rabbits was prepared in the same manner as described by *Kauffmann, Bjørneboe & Vammen* (47) (1938) and by *Mørch* (60) (1942).

Prior to the inoculation of the media with pneumococci, the flasks containing serum broth or ox heart broth were warmed preliminarily by standing in incubator at 37° for about 20 hours. By this, a more rapid growth of the cultures was obtained, and

possibly contaminated flasks could be eliminated. Then, for every 3 liters of broth in the flasks, 5 cc. of defibrinated rabbit blood dilution (20 cc. of defibrinated rabbit blood in 200 cc. distilled water, filtered through filter) was added together with 20 cc. of a 16-18-hour ox heart or serum broth culture, produced as follows:

In the evening a mouse was inoculated with a serum broth culture of the capsulated pneumococcus strain which was to be employed in the experiment. Next morning when the mouse was ill or dead, autopsy was performed and cultures from the heart's blood were made in ox heart's broth or serum broth and on blood agar plate for control of the purity. After incubation at 37° for 24 hours the broth culture from the heart's blood was examined for capsular swelling with homologous immune serum, and subcultures were made on blood agar plate. If the microscopic examination showed it was a pure culture, the flasks were inoculated as mentioned above, and the blood agar plate was made for control of the purity.

When the flasks showed good growth—as a rule after 8-12 hours—a blood agar plate was inoculated for control of the purity and examined for capsular swelling in the same way as the culture from the heart's blood. Then the broth culture in the flasks was centrifuged either for 4-6 hours in a large centrifuge with six metal cups at a rate of 3000 revolutions per min.—it could take 3 liters of culture at a time (production of vaccine for the experiments)—or in a rapid milk centrifuge which could centrifuge 100 liters of culture in about 3 hours (production of vaccine for immunization).

The R vaccines were prepared in the same manner as the S vaccine but with omission of the mouse passage, and the broth flasks were inoculated directly with the ox heart broth culture; and the examination for capsular swelling was carried out with the homologous S immune serum.

For the experiments the centrifugate was transferred into sterile ampullas of 1 cc. (in 10 experiments), 5 cc. (in 152 experiments), 10 cc. (in 4 experiments), or 20 cc. (in 4 experiments), or to thick-walled sterile test-tubes (in 3 experiments) that were sealed and then immersed in water bath kept automatically at

the temperature desired. In concentration, 1 cc. of the sediment corresponded to about 100 cc. of culture.

The number of pneumococci in the vaccine was found by diluting 0.1 cc. of the centrifugate with saline till the dilution had the same density as a known suspension of bacteria, Standard 8, containing 2500 million bacteria per cc.; the number of pneumococci would then be 10 times the degree of dilution multiplied by 2500. The number of pneumococci in the vaccines was estimated altogether 167 times, and the average was 792455 millions per cc., with variations from 250000 to 2750000 millions, but most often between 700000 and 800000 millions.

For immunization the pneumococci in the whole culture were killed by addition of 2 % formalin (33 %), whereafter the flasks were incubated at 37° for 24 hours and then centrifuged in the milk separator. The compact viscous sediment was suspended in Sørensen buffer solution (pH 7.38), and after addition of 1 % formalin it was poured into flasks and stored in ice-box.

SSS (specific soluble substance) was produced by autolysis from the pneumococci with 5-6 cc. of ox bile per 100 cc. of broth culture. The autolyzed culture was acidified by addition of 1 cc. of 50 % acetic acid per cc. of culture, and heated to boiling. After cooling, the mixture was neutralized with sodium hydroxide and centrifuged for 20 min. The clear supernatant fluid contained the type-specific carbohydrate, which was precipiated with absolute alcohol and dried in desiccator or incubator. SSS in dilution 1:60000 precipitated the homologous serum.

In the following, a figure affixed to SSS gives the type from which the specific carbohydrate was obtained.

5. *Immune Serum.*

Immune sera were produced by intravenous injection of vaccine in white rabbits.

At the commencement of the immunization the rabbits weighed 2-3 kg., but they gained in weight during the immunization. The immunization was carried out exclusively with formalin vaccine of the same density as Standard 1, *i. e.*, 1000-2000 million pneumococci per cc. Prior to the injection, the vaccine was

warmed to 37°. No strict schema of dosage could be set up, as the toxicity of the vaccine and the sensitiveness of the rabbits had to be taken into consideration, but the general procedure consisted in 3 weekly injections with the following doses:

1'	week:	0.25	cc. -	0.5	cc. -	0.5	cc.,	Standard 1
2'	»	1.0	»	1.0	»	1.0	»	»
3'	»	1.5	»	1.5	»	1.5	»	»
4'	»	2.0	»	2.0	»	2.0	»	»
5'	»	2.0	»	2.0	»	2.0	»	»

and so on.

If the animals stood the injections well, the dose was sometimes increased. But as a rule 2 cc. per injection was the greatest dose.

After immunization from 6 to 8 weeks the agglutinative and capsular titers of the serum were determined. If the titers were sufficiently high, the rabbits were bled by heart puncture, and the blood was left standing for coagulation at room temperature for a couple of hours. Then the clot was loosened from the wall of the tube and broken up, whereafter the blood was placed in ice-box (4°) for 24 hours. Then the serum was poured off and centrifuged and filtered; 0.5 % phenol (10 % of a 5 % phenol solution) was added to the serum under gentle shaking.

Now the capsular and agglutinative titers were determined. Here »titer« means the reciprocal value of the highest serum dilution giving respectively distinct capsular swelling with all the pneumococci and macroscopic flocculation. In the reading of the agglutination, distinction was made between 3 degrees:

1. ++ = coarse floccules or flakes.
2. + = smaller but macroscopic floccules.
3. (+) = floccules visible only under magnifying glass.

For determination of the titers, only the first two degrees were taken into account.

The capsular and agglutinative titers were determined simultaneously. Into a series of Widal tubes, the various serum dilutions were pipetted off, beginning—from left to right—with un-

diluted serum or dilution 1:10; and in the following tubes, the double dilution. To each tube was added 0.2 cc. of serum and 0.2 cc. of pneumococcus vaccine with a density of about 100 million pneumococci per cc. Thus the tubes contained respectively the serum dilutions 1:2, 1:4, 1:8, etc., and 1:20 1:40, 1:80 etc. As control test for self-agglutination of the vaccine, 0.2 cc. of saline was added to a tube containing 0.2 cc. of vaccine. The tubes were shaken well, and from each tube a loopful of the mixture was examined under the microscope, and the highest serum dilution giving a distinct capsular swelling was noted. Then the tubes were placed in water-bath at 50° for 20 hours, whereafter the agglutination was read.

6. *Production of R Pneumococci.*

In my experiments the R pneumococci were produced by repeated subcultivation of the corresponding S pneumococci in homologous immune serum. At first the pneumococci were grown in undiluted homologous serum with a capsular and agglutinative titer of about 640, but after a few passages, further subcultivation was difficult. For all my experiments, therefore, the pneumococci were grown in about 14 % homologous immune serum, 1 cc. of concentrated serum being added to a test-tube containing about 6 cc. of serum broth. To begin with, subcultures in immune serum were made daily, later only every other day, and, for control of the purity, blood agar plates were inoculated at the same time.

7. *Tests for Sterility of the Vaccine.*

Examination of the vaccine for sterility alone was performed altogether 37 times: in 10 experiments with 1 cc. ampullas, in 19 experiments with 5 cc. ampullas, and in 4 experiments with 10 and 20 cc. ampullas. In all the other experiments in vivo and in vitro the tests for sterility were made together with the experiments on transformation of type, so that in each experimental series about 5 cc. of vaccine was used exclusively for examination of the sterility.

The culture media mentioned in Section 1 were inoculated with vaccine in amounts from 1 loopful to 1.25 cc., and then incubated at 37°. From the fluid media, subcultures (with 1 loopful of culture) on blood agar plate or in serum broth or both were made as a rule daily for 8 days; they were incubated and examined on the following day. A few times subcultures were made daily for 14 days or only every other day for 8 days. The blood agar plates inoculated directly from the vaccine were incubated at 37° for 9 days.

Besides being incubated at 37°, in some tests the subcultures were placed in a glass jar where the air contained 5 % CO_2, produced in the following way: In the glass jar covered by a lid, which was made airtight by means of vaseline on the edge of the jar, 10 g. of sodium bicarbonate was placed on the floor, and a small test-tube containing 5 cc. of concentrated sulphuric acid was fastened in such a manner that the sulphuric acid would flow out on the sodium bicarbonate when the container was tilted a little; and then CO_2 developed in amount corresponding to 5 % of the air in the jar. Every day or every other day for 8 days the cultures were taken out of the jar, and subcultures were made on 10 % blood agar plates which were incubated at 37° in ordinary atmosphere for 24 hours and then examined.

Some of the inoculated media were incubated anaerobically at 37°, the air in the glass jar being evacuated by means of a suction pump, and after 8 days the tubes were again taken out of the jar, and 1 loopful of culture was plated on 10 % blood agar, which then was incubated for 24 hours at 37°.

A few sterility experiments were carried out after the method given by *S. L. Ørskov* (93) for cultivation of typhoid, paratyphoid, paradysentery and colon bacilli: test-tubes with 12 cm. high agar —some of them with an addition of 2 cc. of 10 % Wollstein blood extract broth—were melted in water-bath and cooled to 50°; then 1.25 cc. of warmed vaccine was added and the tubes were incubated at 37°. In the following 8 days subcultures were made from the deeper as well as superficial layers on 5 % and 10 % blood agar plates and in serum broth, which then were incubated at 37° for 24 hours.

Finally, a few experiments were carried out with *Tarozzi* (85)

medium, the vaccine being added to test-tubes with serum broth containing small pieces of liver and kidney from rabbit or guinea-pig. The culture was covered by a 2 cm. layer of sterile liquid paraffin and every day for 8 days a loopful of the culture was spread on a 5 % blood agar plate, while another loopful was transferred to serum broth. The subcultures were then incubated for 24 hours.

In 4 of the experiments with 6 ampullas of 5 cc., one ampulla was each time put into 100 cc. of 5 % blood agar, another ampulla into 100 cc. of agar, which first had been melted and then cooled to 50°; the mixture was then poured out into 4 sterile Petri dishes. Thus each agar plate contained 1.25 cc. of vaccine, which was mixed thoroughly with the medium, and in this way cultivation under slightly anaerobic as well as aerobic conditions was obtained.

Further, in another experiment, after heating, all the vaccine from 10 and 20 cc. ampullas was pooled with 200 cc. of melted 10 % blood agar and the mixture was poured out into 6 Petri dishes, so that each dish contained respectively 1⅔ and 3⅓ cc. of vaccine. Three blood plates of each kind were placed in ice-box for 3 days and then incubated at 37° for 6 days. Two blood plates of each kind were at once incubated for 9 days. The last plate of each kind was inoculated with a streak culture of Coccus 858 B and incubated at 37° for 9 days. This coccus altered the hemoglobin in its vicinity to a still unknown substance which had a promoting effect on the growth of the other bacteria.

Sterility experiments were carried out not only in vitro but also in vivo. The heated vaccine was injected subcutaneously over the left groin in white mice — either alone or together with cultures of streptococci, staphylococci, colon bacilli or killed R pneumococci. The amount of vaccine varied from 0.1 cc. to 1.0 cc. and the amount of the other bacterial cultures from 0.25 cc. to 0.75 cc. Furthermore, the vaccine was injected also together with a solution of mucin, the mucin being injected either subcutaneously (½-1 hour before the vaccine or at the same time) or intraperitoneally (½-1 hour before the vaccine).

Finally the vaccine was injected subcutaneously on mice ½-1 hour after intraperitoneal injection of 0.5-1.0 cc of 10 % alcohol.

When the mice died, they were autopsied and examined in the manner mentioned in the next paragraph. Most of the mice survived the injection and were killed 14-20 days after, when they were autopsied and examined in the usual manner.

8. *Experiments on Transformation of Type in vivo.*

The experiments were carried out on white mice, which were given a subcutaneous injection in the left groin of vaccine and R culture which was examined beforehand in the sera produced with the two S types corresponding to the vaccine and the R form. In addition, the purity of the R culture was examined by plating on 10 % blood agar.

When the mice died or, if surviving the injection, were killed 14-16 days after, they were autopsied. They were stretched out on a board with 4 large pins; the abdomen was washed with 96 % alcohol, an incision was made in the skin with sterile scissors and pincers, and the skin was loosened (in the subcutis) over the entire anterior aspect of the abdomen and thorax; then the skin was divided in the midline, giving two flaps, one on each side, that could be turned back. At the site of the injection, on the subcutis, the dead mice showed as a rule an abscess, while the survivors presented some fibrous adhesions or a wound, as the skin here had undergone necrosis and ulceration, leaving the peritoneum exposed; cultures in serum broth and on 10 % blood agar plate were made from this area with a sterile loop. With fresh sterile instruments the sternum was removed and the heart pulled forwards. The tip of the heart was cut off; and by means of a sterile loop a little of the heart's blood was transferred to a tube with serum broth, and also spread on 10 % blood agar plates. The peritoneal cavity was opened and a sterile loop was introduced between the intestines—for inoculation of 10 % blood agar plate with the peritoneal fluid. After this, some sterile physiological salt solution was withdrawn with a sterile Pasteur pipette and used to wash the peritoneum thoroughly a couple of times, and then the saline was removed again by means of the Pasteur pipette, and transferred to a sterile thick-walled test-tube.

The two serum broth tubes and the three 10 % blood agar plates were incubated at 37° for 24 hours, whereafter the contents of the broth tubes were examined for capsular swelling with the sera for the two S types corresponding to the vaccine and the R pneumococcus. Numerous other pneumococcus colonies on the blood agar plates were examined with the same sera. From suspicious colonies subcultures were made on blood agar plate or on serum broth or on both, and after incubation at 37° overnight they were examined for capsular swelling. On the lavage the Neufeld reaction was performed with the sera of the two S types.

9. *M & B Experiments.*

Some pneumococcal strains are sensitive to sulfapyridine, M & B 693 (M & B), while others are resistant. The sensitivity of the pneumococci to M & B was determined after the method given by *Schmith* (79) (1941).

A stock solution of M & B 1 : 5000 was made, for instance, by dissolving 200 mg. of M & B in 1000 cc. of serum broth, and 8 different dilutions were made in serum broth. As M & B is only slightly soluble in serum broth, M & B was placed in ordinary broth which was heated in boiling water. When the drug was completely dissolved, the broth was cooled and 5 % inactivated ox serum was added. From this stock solution the other solutions of M & B were prepared as follows:

250	cc.	stock	sol.	+	250	cc.	serum	broth	=	M&B	concentr.	1:10000
125	»	»	»	+	375	»	»	»	=	»	»	1:20000
62.5	»	»	»	+	437.5	»	»	»	=	»	»	1:40000
31.3	»	»	»	+	468.7	»	»	»	=	»	»	1:80000
15.6	»	»	»	+	484.4	»	»	»	=	»	»	1:160000
7.8	»	»	»	+	492.2	»	»	»	=	»	»	1:320000
3.9	»	»	»	+	496.1	»	»	»	=	»	»	1:640000

These 8 M & B solutions were filtered through Berkefeld filter, poured into test-tubes, about 5 cc. in each tube, and stored in ice-box at 4° until use. The tubes with the decreasing M & B concentrations were numbered from 1 to 8 as follows:

Tube	1	M&B	concentration	1:5000
»	2	»	»	1:10000
»	3	»	»	1:20000
»	4	»	»	1:40000
»	5	»	»	1:80000
»	6	»	»	1:160000
»	7	»	»	1:320000
»	8	»	»	1:640000

When the sensitivity of a pneumococcus strain to M & B had to be determined, 0.05 cc. of a 24-hour culture diluted 1 : 2000 was added to a series of M & B tubes. After shaking, the tubes were incubated at 37° for 24 hours. Then it was recorded which tubes were cloudy from bacterial growth and which were clear on account of the bacteriostatic or bactericidal property of M & B, and the tubes were again incubated for 24 hours and read once more, with recording of the clear and cloudy tubes.

By numbering the tubes in each series from 1 to 8, the sensitivity of the pneumococcus strains to M & B could be expressed by the number of the tube with the highest concentration of M & B which remained clear after 24 and 48 hours. Thus, for instance, sensitivity 6 : 4 signified that tube 6 with M & B concentration 1 : 160000 was clear 24 hours after addition of the pneumococcus culture and tube 4 with M & B concentration 1 : 40000 was clear 48 hours after the inoculation. The second reading, after 48 hours, balanced the small variations that might appear on repeated examination of the same strain.

Schmith (79) further gave a method by which a primarily sensitive pneumococcus type could be made resistant to M & B. A series of M & B tubes were inoculated with the sensitive pneumococcus strain. After incubation for 48 hours at 37° a new series of M & B tubes were inoculated with the first cloudy tube in the preceding series. Now the resistance proved to be increased by one or two tubes. By continued passages through M & B solutions—every time with subcultivation from the first cloudy tube in the preceding series—the resistance of the pneumococci was increased till they were refractory to M & B.

The M & B experiments were carried out with an R pneumococcus that was either sensitive or resistant to M & B, together with a vaccine made of an S pneumococcus which respectively was resistant or sensitive to M & B; and in each experiment the sensitivity to M & B was determined for the R and S pneumococci employed. Otherwise the experiments were carried out as outlined in Section 8.

10. *Fermentation Tests.*

My fermentation experiments were carried out with the employment of Hiss' culture medium, which was given by *Hiss, Borden & Knapp* (42) and recommended by *Levy* (57), *Christiansen* (13), *Viktorow, Semzowa & Sinjuschina* (86) and *Jürgens* (46).

The Hiss medium consists in 1 part ox serum and 2 parts distilled water. The substances to be fermented were added in an amount of 0.5 %, and bromocresol purple was used for indicator. pH is adjusted to 7.8; and the color of the medium is a deep blue. During fermentation, acid is formed, and this reduces pH; at the same time the bromocresol purple changes in color from blue to yellow, and the medium coagulates.

Like *Christiansen* (13) and *Jürgens* (46), I have used horse serum for my experiments instead of ox serum. The medium was placed in narrow test-tubes, to a height of 2 cm.

The pneumococcus strain under analysis (serum broth culture) was in the evening injected intraperitoneally into a white mouse on which autopsy was performed next morning. A serum broth culture was made from the heart's blood and incubated at 37° for 12 hours. From this serum broth subcultures were made in Hiss' medium. In the first fermentation experiment, only one loopful of culture was transferred to the subculture, but in most of the experiments one drop of culture was transferred with Pasteur pipette to each tube of medium. The tubes were incubated at 37° and inspected daily for two weeks. If fermentation took place the medium coagulated and turned light yellowish in color. Only distinct changes in color and coagulation were reckoned as positive fermentation. For control, Hiss' me-

dium without any addition of sugar, alcohol or glucocide was inoculated and incubated like the other tubes.

11. *Filtration Experiments.*

The experiments were carried out in the same manner as mentioned in Section 8, but the vaccine was produced by heating in autoclave to 100° for 1 hour, and filtrate of a 24-hour S culture in ox-heart broth was injected together with the R culture and S vaccine. The filtration was carried out with a Chamberland L_2 or L_5 filter.

12. *Transformation Experiments in vitro.*

The experiments were carried out by mixing (in test-tubes) vaccine or, in one experiment, the specific substance of the pneumococci with R culture, anti-R serum or—once—normal swine serum, serum broth or blood broth. In some experiments the tubes were incubated at 37° for 15 days, and every day 1 loopful of culture was spread on a 10 % blood agar plate, and a loopful was transferred to a tube with serum broth. In other experiments, after incubation of the tubes for 24 hours, 1 loopful of culture was transferred to another tube containing the same amount of vaccine, anti-R serum and serum broth or blood broth, and this transfer of culture was repeated on 5-10 successive days; at the same time subcultures were made with one loopful of culture on blood agar plate and in serum broth. The plates and serum broth tubes were incubated at 37° for 24 hours and then examined for the presence of capsulated pneumococci.

13. *Absorption Experiments.*

The absorption experiments were carried out with the same formalin vaccine as was employed for immunization, but here the vaccine was used in a denser suspension (10-20 times denser than Standard 1; for the R pneumococci, up to 50 times denser). Centrifuges tubes of 5 cc. were filled with vaccine, which then was centrifuged for half-an-hour at a rate of 5000 revolutions per min.

In this way the vaccine was centrifuged so strongly that the supernatant fluid was clear and could be poured off. The tube was then filled with the serum that was to be absorbed, and vaccine and serum were mixed thoroughly by means of a Pasteur pipette, then incubated for 2 hours and afterwards placed in ice-box for about 18 hours. After this the mixture was again centrifuged for 30 min. at the same rate as before, and the supernatant clear fluid was pipetted off. The titer of the serum being high, as a rule it was necessary to absorb twice before the absorption was complete. In that case, the serum after the first centrifuging was mixed with an additional tube of centrifuged vaccine of the same type and otherwise treated as in the first absorption.

In order to see whether the absorption was complete the capsular or agglutinative titer of the serum was determined with the type of the absorbed vaccine when it was a question of S or R pneumococci respectively. With R pneumococci, as a rule, 3 absorptions—once even 5 absorptions—were required before the serum was completely exhausted for the antigen of the vaccine.

After the absorption the capsular and agglutinative titers of the absorbed sera were determined for both homologous and heterologous pneumococci, the titration being carried out as mentioned in Section 5.

Chapter II.

STUDIES ON R PNEUMOCOCCI

1. *Stability.*

For my experiments I used R pneumococci of Types 1, 2 and 8; i addition R 3, R 14, R 20, R 22 and R 35 were produced. The number of passages in homologous immune serum required for the conversion of capsulated pneumococci into stable R pneumococci varied with the types. »Stable« R pneumococci means that they do not revert to S pneumococci on passing repeatedly through mice or serum broth. Types 1 and 2 formed stable R pneumococci as soon as after 5 passages in 14 % homologous immune serum, whereas Type 8 formed only unstable R pneumococci even after 83 passages, for on intraperitoneal and subcutaneous injection into white mice R 8 reverted to S 8—though not on cultivation in serum broth. This was also observed by *Baurhenn* (8), who pointed out the difficulty in getting Type 8 to lose its capsule by cultivation in homologous immune serum.

Besides S 8, it was also difficult to make S 22 and S 35 form stable R pneucococci, as R 22 and R 35, formed from the corresponding S pneumococci after 20 homologous immune serum passages, reverted to their homologous S types.

2. *Behavior towards M & B.*

Of Types 1, 2 and 8 the corresponding R pneumococci were formed by cultivation in homologous immune serum, and their sensitivity to M & B was determined. If the capsulated pneumococci were sensitive to M & B, the non-capsular pneumococci

would also be sensitive; if the former were resistant, the R pneumococci would also be resistant. The degree of sensibility was further determined after the R pneumococci had been grown several times in serum broth and also for R cultures obtained from a single colony, but the degree of sensitivity remained unchanged. A sensitive R pneumococcus remained sensitive, and a resistant R pneumococcus remained resistant.

The sensitivity of the pneumococci to M & B is thus a constant property localized to the species-specific part (body) of the pneumococci, not to the type-specific (capsule).

3. *Serology.*

Reimann (76) (1926) claimed that all R pneumococci were serologically identical, with proteins for chief antigen. He arrived at this conclusion for the following 3 reasons: 1) Anti-S 1 serum agglutinated not only S 1 but also R pneumococci regardless from which capsulated pneumococcus the R pneumococci originated; it also precipitated autolyzed R pneumococci and solutions of pneumococcal protein. 2) Anti-R serum contained agglutinins for heterologous as well as homologous R pneumococci and precipitins for autolysates of both S and R pneumococci. 3) Absorption with the homologous and heterologous R strain removed agglutinins for R pneumococci and precipitins for pneumococcal protein from anti-R serum.

Griffith (37, 38) and *Baurhenn* (8) likewise thought that all R pneumococci were identical as one R strain could be transformed into heterologous S pneumococci. But none of the authors have thoroughly investigated the serological identity of the R pneumococci, as they have not carried out cross-absorption between the various R pneumococci and their sera.

In order to prove that pneumococcus A is identical with pneumococcus B, vaccine A must be able completely to empty serum B of B agglutinins, and vaccine B must be able completely to empty serum A of A agglutinins.

If only one series of such experiments is carried out, nothing can be said about the serological identity of the two pneumococcus types. If vaccine A empties serum B completely, this

may be due either to complete identity of the pneumococci A and B or to the circumstance that pneumococcus A contains all the antigens of pneumococcus B plus some others. If, on the other hand, vaccine B also empties serum A, it can only be because A and B are identical.

The following experiments (Table 1) were aimed to show that the R pneumococci were serologically identical. The experiments were carried out with R vaccine and the corresponding serum of Types 1, 2 and 35. All the R vaccines formed stable suspensions in the dilution here employed, and thus they were not self-agglutinable. The three R sera were absorbed with both homologous and heterologous R vaccines, and the agglutinative titer was determined with the 3 R vaccines and corresponding S vaccines both before and after the absorption. In addition, the agglutinative titers were determined for the three S and R vaccines and sera mutually.

All sera were absorbed twice.

From Table 1 it will be noticed that the R pneumococci of Types 1, 2 and 35 are serologically identical as their sera are emptied completely of agglutinins by cross-absorption.

In subsequent experiments it was further demonstrated that R 8 is serologically identical with R 1 and R 2.

When capsulate pneumococcal sera give very faint agglutination in the first tube with homologous and heterologous R pneumococci, it is due to the presence of agglutinins for the pneumococcus protein in the anti-S serum.

From one of the new-formed Type 2 and Type 8 pneumococci (mentioned in Chapter V) the corresponding R forms were produced, and the following experiments were aimed to show whether the R pneumococci formed in this way were serologically identical with the R 1 pneumococci employed for the experiment.

The experiment was carried out with R vaccine and corresponding sera of Types 1, 2 and 8 and the new-formed Types 2 and 8, all five R sera being absorbed both with homologous vaccine and heterologous; and the agglutinative titer was determined by means of the five R vaccines and the corresponding S vaccine both before and after the absorption. Furthermore,

TABLE 1

Vaccine	Pneumococcus sera														
	R 1				R 2				R 35				S 1	S 2	S 35
	non-abs.	abs. with			non-abs.	abs. with			non-abs.	abs. with					
		R 1	R 2	R 35		R_1	R_2	R 35		R 35	R 1	R 2			
R 1	640	0	0	0	640	0	0	0	640	0	0	0	20	0	20
R 2	1280	0	0	0	1280	0	0	0	1280	0	0	0	20	20	20
R 35	320	0	0	0	320	0	0	0	640	0	0	0	0	0	20
S 1	0	0	0	0	0	0	0	0	0	0	0	0	1280	0	0
S 2	0	0	0	0	0	0	0	0	0	0	0	0	0	640	0
S 35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	640

R 1 = non-capsulated pneumococcus Type 1.

S 1 = capsular pneumococcus Type 1.

The figures give the agglutination titer.

the agglutination titers were determined for the five S and R vaccines and sera mutually.

As R 2 of the new-formed Type 2 and R 8 were self-agglutinating in the dilution employed for titration, the titration was carried out with a fresh 18-hour culture in serum broh with addition of 0.5 % formalin, and, after standing for 30 min. in incubator at 37°, it was diluted with physiological salt solution until the culture had the same density as the vaccine employed for the titration.

Most of the sera were absorbed twice with the vaccine, a few of them 3 times; and R 1 was absorbed with R 8 of the new-formed Type 8 no less than 5 times before the agglutinins for the type of the vaccine were removed.

From Tables 2 and 3 it will be noticed that R 8 also is serologically identical with R 1 and R 2, as their sera are completely emptied of agglutinins by cross-absorption. In addition, R 1 is seen not to undergo any serological change in the process of transformation, as R 2 (1 → 2) and R 8 (1 → 8) remain serologically identical with R 1.

It is further seen that R 8 and R 8 (1 → 8) agglutinate very faintly with S 8 and S 8 (1 → 8). This is due to the fact that the two R pneumococci have not been stable R forms but contained a little capsular substance. For the same reason, serum R 8 and R 8 (1 → 8), absorbed with vaccine R 1, gave a very faint reaction with the capsulate Type 8 pneumococci, as all agglutinins for the R pneumococci are removed by the absorption, but not the type-specific agglutinins. On the other hand, serum R 8 and R 8 (1 → 8), absorbed with R 8 (1 → 8) and R 8, do not react with vaccine S 8 as here both the species-specific and the type-specific agglutinins are removed.

That the 5 S sera agglutinate with heterologous R pneumococci as well as homologous is due to the presence of agglutinins for the pneumococcal protein and, in the case of Type 2 and Type 8, to the circumstance that R 2 (1 → 2) and R 8 (1 → 8) still contained a little capsular substance.

In the following experiment both anti-S 1 and R 1 serum were absorbed with vaccine S 1 and R 1, and the agglutination titer was determined with homologous and heterologous S and R

TABLE 2

Vaccine	Pneumococcus sera																	
	R 1						R 2						R 2 (1➤2)					
	non-abs	absorbed with					non-abs.	absorbed with					non-abs.	absorbed with				
		R 1	R 2	R 2 (1➤2)	R 8	R 8 (1➤8)		R 1	R 2	R 2 (1➤2)	R 8	R 8 (1➤8)		R 1	R 2	R 2 (1➤2)	R 8	R 8 (1➤8
R 1	640	0	0	0	0	0	640	0	0	0	0	0	640	0	0	0	0	0
R 2	1280	0	0	0	0	0	1280	0	0	0	0	0	640	0	0	0	0	0
R 2 (1—➤2)	640	0	0	0	0	0	640	0	0	0	0	0	1280	0	0	0	0	0
R 8	160	0	0	0	0	0	160	0	0	0	0	0	160	0	0	0	0	0
R 8 (1—➤8)	640	0	0	0	0	0	640	0	0	0	0	0	320	0	0	0	0	0
S 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S 2 (1—➤2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S 8 (1—➤8)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

R 2 (1—➤2) = non-capsulated pneumococci obtained from the new-formed Type 2.

S 2 (1—➤2) = capsular Type 2 pneumococci of the new-formed Type 2.

TABLE 3

Vaccine	Pneumococcus sera																
	R 8						R 8 (1➤3)						S 1	S 2	S 2 (1➤2)	S 8	S 8 (1➤8)
	non-abs.	absorbed with					non-abs.	absorbed with									
		R 1	R 2	R 2 (1➤2)	R 8	R 8 (1➤8)		R 1	R 2	R 2 (1➤2)	R 8	R 8 (1➤8)					
R 1	320	0	0	0	0	0	640	0	0	0	0	0	20	0	20	0	0
R 2	640	0	0	0	0	0	1280	0	0	0	0	0	20	20	0	0	0
R 2 (1➤2)	320	0	0	0	0	0	640	0	0	0	0	0	0	20	40	0	20
R 8	320	0	0	0	0	0	160	0	0	0	0	0	0	0	0	0	40
R 8 (1➤8)	160	0	0	0	0	0	1280	0	0	0	0	0	0	0	0	20	0
S 1	0	0	0	0	0	0	0	0	0	0	0	0	1280	0	0	0	0
S 2	0	0	0	0	0	0	0	0	0	0	0	0	0	640	320	0	0
S 2 (1➤2)	0	0	0	0	0	0	0	0	0	0	0	0	0	640	640	0	0
S 8	40	<20	20	20	0	0	20	<20	<20	<20	0	0	0	0	0	640	320
S 8 (1➤8)	40	<20	<20	0	0	0	0	0	0	0	0	0	0	0	0	160	320

R 8 (1 ➤8) = non-capsulated pneumococci obtained from the new-formed Type 8.

S 8 (1 ➤8) = capsular Type 8 pneumococci of the new-formed Type 8.

pneumococci before and after the absorption. All the absorptions were performed twice before the titration. The findings are given in Table 4.

TABLE 4

Vaccine	Pneumococcal sera					
	R 1			S 1		
	non-absorbed	absorbed with R 1	absorbed with S 1	non-absorbed	absorbed with S 1	absorbed with R 1
R 1	640	0	0	20	0	0
R 2	1280	0	0	20	0	0
S 1	0	0	0	1280	0	320
S 2	0	0	0	0	0	0

The experiment was further carried out with Types 2 and 35, and the results corresponded perfectly to those obtained with Type 1.

From Table 4 it will be noticed that anti-R serum is emptied of agglutinins for R pneumococci by absorption with the homologous R pneumococci or the homologous S pneumococci, whereas the anti-S serum is emptied of agglutinins only by absorption with the homologous S pneumococci, not with the homologous R pneumococci. In the latter case the agglutinative titer falls 1 or 2 tubes, owing to the fact that sera are weakened by absorption.

Besides the agglutinative titer, also the capsular titer for anti-S serum was determined by employment of homologous S pneumococci both before and after the absorption with R and S pneumococci, and as a rule it was found to be 1 or 2 tubes higher than the agglutinative titer. On absorption with the homologous S pneumococci, also the capsular agglutinins were removed.

Chapter III.

STUDIES ON THE STERILITY OF THE VACCINE

1. *Sensitivity of Pneumococci to Heat.*

In order to see to which temperature the pneumococci had to be heated in order to be killed, Types 1, 2 and 8 were heated under water in sealed ampullas of 5 cc. for 20 min. to 47°, 50°, 52°, 55°, 57° and 60°. The pneumococci were heated partly as serum broth and ox heart broth cultures, partly as centrifugates of these cultures, prepared in the same way as vaccine (see Chapter I, 4), and partly as suspensions in physiological salt solution, a very small portion of the centrifugate being suspended in saline unto the density of a 10-12-hour broth culture.

The heating of the three types was performed at least twice at each of the six temperatures, and each time with 5 cc. After the heating, 1.0 cc. was transferred to test-tubes with blood broth, horse serum broth, ox serum broth, ox heart broth and Truche, and 10 % blood agar plate was inoculated with 0.2 cc.; all the cultures were incubated at 37°.

On the following 8 days, one loopful of culture was daily transferred from the test-tubes to 10 % blood agar plates, which after incubation for 24 hours were examined for pneumococci. As soon as growth was noticed in the tube, the subcultivation on blood agar plates was discontinued. The tubes which turned out to be contaminated within the eight days were discarded and not taken into account in the following three tabulations of results.

The blood agar plates inoculated immediately after the heat-

ing of the cultures were incubated for eight days and examined daily for growth of pneumococci.

Tables 5, 6 and 7 give the results obtained in the experiments carried out with Types 1, 2 and 8.

TABLE 5

Temperature	Pneumococcus Type 1					
	Serum broth			Ox heart broth		
	culture	conc.	dilut.	culture	conc.	dilut.
47°	12 +	12 +	12 +	7 + 4 —	12 + 1 —	18 + 1 —
	100 %	100 %	100 %	63,6 %	92,3 %	94,7 %
50°	12 +	12 +	10 + 1 —	9 —	8 —	10 + 3 —
	100 %	100 %	90,9 %	0 %	0 %	76,9 %
52°	5 + 8 —	10 + 2 —	12 —	10 —	8 —	11 —
	38,5 %	83,3 %	0 %	0 %	0 %	0 %
55°	11 —	13 —	9 —	11 —	1 + 11 —	10 —
	0 %	0 %	0 %	0 %	8,3 %	0 %
57°	12 —	9 —	8 —	11 —	12 —	12 —
	0 %	0 %	0 %	0 %	0 %	0 %
60°	8 —	13 —	8 —	12 —	10 —	9 —
	0 %	0 %	0 %	0 %	0 %	0 %

dilut. = pneumococci after centrifuging suspended in saline.
conc. = centrifugate of the culture.
+ after a figure = No. of subcultures with growth of pneumococci within 8 days.
— after a figure = No. of subcultures without growth of pneumococci within 8 days.
The figures under the No. of cultures = percentage of the No. of subcultures showing growth of pneumococci.

When growth of pneumococci occurred, it most often appeared within the first 24 hours, a few times within 2 days, and never later.

From Tables 5, 6 and 7 it will be noticed that on heating to a certain temperature the percentage of surviving pneumococci

TABLE 6

Temperature	Pneumococcus Type 2					
	Serum broth			Ox heart broth		
	culture	conc.	dilut.	culture	conc.	dilut.
47°	17 + 100 %	12 + 100 %	12 + 100 %	17 + 100 %	12 + 100 %	12 + 100 %
50°	5 + 5 — 50 %	7 + 9 — 43,8 %	12 — 0 %	8 — 0 %	5 + 4 — 55,6 %	11 — 0 %
52°	11 — 0 %	12 — 0 %	1 + 12 — 7,7 %	11 — 0 %	10 — 0 %	9 — 0 %
55°	1 + 10 — 9,1 %	12 — 0 %	13 — 0 %	11 — 0 %	11 — 0 %	13 — 0 %
57°	1 + 6 — 14,3 %	11 — 0 %	10 — 0 %	9 — 0 %	10 — 0 %	8 — 0 %
60°	10 — 0 %	11 — 0 %	12 — 0 %	8 — 0 %	8 — 0 %	13 — 0 %

in ox heart broth is lower than in serum broth. This means that pneumococci growing in serum-containing media have to be heated to a higher temperature in order to be killed than do pneumococci growing in ox heart broth.

On this point my experiments are in agreement with the observations made by *Händel & Lange* (40).

It is further seen that in order to get all the pneumococci killed the centrifugate has to be heated to a higher temperature than the culture itself, which again has to be heated to a higher temperature than the pneumococcal suspension in physiological salt solution. This fact is quite conspicuous after growth in serum broth, less distinct after growth in ox heart broth. This means that the denser the pneumococcal suspension, the higher is the temperature required for the killing of all the bacteria.

In addition, the tables show that pneumococci grown in ox heart broth as a rule are killed on heating to 55° for 20 min.; only in one instance did the blood agar plate show growth of Type 1 after heating to 55° for 20 min. Pneumococci grown in

TABLE 7

Temperature	Pneumococcus Type 8					
	Serum broth			Ox heart broth		
	culture	conc.	dilut.	culture	conc.	dilut.
47°	12 +	12 +	12 +	10 + 2 —	8 + 1 —	7 + 3 —
	100 %	100 %	100 %	83,3 %	88,9 %	70 %
50°	11 + 1 —	12 +	9 + 3 —	6 + 5 —	6 + 7 —	5 + 2 —
	91,7 %	100 %	75 %	54,5 %	46,2 %	71,4 %
52°	9 + 2 —	8 +	6 + 4 —	4 + 5 —	10 —	2 + 8 —
	81,8 %	100 %	60 %	44,4 %	0 %	20 %
55°	8 —	3 + 6 —	9 —	11 —	12 —	10 —
	0 %	33,3 %	0 %	0 %	0 %	0 %
57°	11 —	13 —	8 —	8 —	14 —	14 —
	0 %	0 %	0 %	0 %	0 %	0 %
60°	8 —	12 —	12 —	12 —	11 —	10 —
	0 %	0 %	0 %	0 %	0 %	0 %

serum broth are nearly always killed on heating to 57° for 20 min.; only in one instance was this treatment followed by growth of Type 2.

The experiments show that Types 1, 2 and 8 do not differ distinctly in their resistance to heat. Perhaps the percentages of growth are somewhat higher for Type 8 than for Types 1 and 2 both after cultivation in serum broth and in ox heart broth, indicating that Type 8 is more able to stand heating than are Types 1 and 2.

After heating to 60° for 20 min. no instance of surviving pneumococci was seen—neither after growth in serum broth nor in ox heart broth.

2. *Tests for Sterility in vitro.*

Altogether 2917 tests for sterility in vitro were carried out with Types 1, 2, 6, 8, 14, 20, 22 and 35. Of this total, 2696 tests or 92.4 % were performed with pneumococci grown in ox heart

broth, the remaining 221 or 7.6 % with pneumococci grown in serum broth.

Considering the individual types, most tests for sterility were carried out with vaccine 2, a total of 1150 tests (39,4 %); next comes vaccine 1 with 887 tests (30.4 %), then vaccine 8 with 531 tests (18.2 %), vaccine 35 with 156 tests (5.3 %), vaccine 22 with 121 tests (4.2 %), vaccine 6 with 48 tests (1.7 %), and vaccines 14 and 20 with 12 tests (0.4 %) each.

Of the total 2917 tests, 2857 (97.9 %) were performed with vaccine prepared after heating to 60° for 20 min., while 60 tests (2.1 %) were carried out with vaccine heated to 60° for 15, 25, 30, 40, 50, 60 or 90 min., or to 100° for 60 min.

Of the culture media mentioned in Chapter I, 1, flasks with 100 cc. of serum broth were inoculated 78 times, with 100 cc. of ox heart broth 64 times, test tubes with 6 cc. of serum broth 611 times, with 6 cc. of serum broth plus 0.15-0.30 % glucose 104 times, with 6 cc. of 10 % horse serum broth 458 times, with 6 cc. of ox heart broth 199 times, with 6 cc. of 5 % blood broth 453 times, with 6 cc. of Truche 230 times, with 6 cc. of Martin broth 40 times, with 6 cc. of trypsin broth 19 times, with 6 cc. of thin agar (sometimes with addition of Wollstein's blood extract broth) 40 times, 10 % blood agar plate 534 times, 5 % blood agar plate 67 times, and agar plate 8 times. Further, 4 tubes were inoculated after *S. L. Ørskov*'s method (93), and 8 tubes after *Tarozzi*'s method (85). The amount of vaccine used in each test varied from 1 loopful to 3⅓ cc.

Of these tests for sterility, 1 flask with 100 cc. of serum broth, 2 test tubes with serum broth, 4 with horse serum broth, 5 with Martin broth, 4 with trypsin broth, and 19 blood agar plates were incubated at 37° in CO_2 atmosphere; 1 flask with 100 cc. of ox heart broth, 1 flask with 100 cc. of serum broth, 5 tubes with serum broth, 15 with thin agar, and 2 blood agar plates were incubated under anaerobic conditions. All the other culture media were incubated in ordinary atmosphere.

From each of all these cultures, subcultures were made daily for 8 days with 1 loopful on 10 % or 5 % blood agar plate or in serum broth, or both on blood agar plate and in serum broth, which after incubation were examined for sterility. From some

cultures, subcultures were made daily for 14 days, while in a few instances subcultures were made only every other day.

In no instance was growth of pneumococci demonstrated. In 24.3 % of the cultures contamination resulted from the daily transfers, but no living pneumococci were demonstrated prior to the contamination.

3. *Tests for Sterility in vivo.*

Altogether 533 tests for sterility in vivo were carried out with the same types as the tests in vitro. Of this total, 457 tests or 85.7 % were performed with pneumococci grown in ox heart broth, while the remaining 76 tests or 14.3 % were carried out with pneumococci grown in serum broth.

As to the individual types, most tests were made with vaccine 2, a total of 234 tests (43.9 %); next comes vaccine 1 with 156 tests (29.3 %), then vaccine 8 with 94 tests (17.6 %), vaccine 6 with 21 tests (3.9 %), vaccine 35 with 14 tests (2.6 %), vaccine 22 with 10 tests (1.9 %), and vaccines 14 and 20 with 2 tests (0.4 %) each.

Of the 533 tests in vivo 487 (91.4 %) were carried out with vaccine prepared after heating to 60° for 20 min., while 46 tests (8.6 %) were performed with vaccine heated to 60° for 15, 25, 30, 40, 50, 60 or 90 min., or to 100° for 60 min.

As mentioned in Chapter I, 7, the mice were inoculated subcutaneously, either with vaccine alone (223 tests = 41.8 %) in amounts from 0.10 to 1.0 cc., or together with colon bacilli (218 times = 40.9 %) in the form of undiluted culture (2 times) or in dilution 1:10 (178 times), 1:20 (20 times), 1:30 (10 times) and 1:100 (8 times) in doses of 0.25-0.75 cc. In addition, the vaccines were injected together with undiluted strept. fæcalis 905 culture (20 times = 3.8 %), staph. aureus culture (4 times = 0.7 %) undiluted (2 times) or diluted 1:10 (2 times), or with R pneumococci killed by heating to 100° (10 times = 1.9 %). The streptococcus, staphylococcus and killed R pneumococcus cultures were injected in amounts of 0.25 cc. Further, the vaccine was injected also together with the specific soluble substance of the pneumo-

cocci (5 times = 0.9 %), mucin (36 times = 6.8 %), subcutaneously (20 times), or intraperitoneally (16 times) (*J. Ørskov* (92)), pneumococcus serum Type 14 (2 times = 0.4 %), and alcohol (15 times = 2.8 %), which was injected intraperitoneally (*Stillmann* (82)). SSS, mucin, pneumococcus serum Type 14 and alcohol were injected in doses from 0.25 cc. to 1.0 cc.

Of the 533 mice, 154 (28.9 %) died within two weeks, the remaining 379 mice survived the infection and were killed on the 14'-16' day. On all the mice, autopsy was performed in the usual way as mentioned in Chapter I, 7, cultures being taken from the subcutis, heart and peritoneum on blood agar plate and in serum broth, and examined for the presence of pneumococci after incubation at 37° for 24 hours.

In one of the tests for sterility in vivo with vaccine 8, one mouse was killed, 1, 2, 3, 4, 5, 6, 7, 9 and 11 days after subcutaneous injection of 0.75 cc. of vaccine. Besides examination of the mice in the usual manner the regional lymph gland, inguinal gland on left side, spleen and liver were removed and minced, then spread on blood agar plate and transferred to serum broth which were examined as usual, after incubation for 24 hours at 37°. The idea was that possibly surviving pneumococci of the vaccine would be transported by way of the lymphatics to the regional lymph gland, liver and spleen and there killed because of the decrease in virulence on heating before they would have time to multiply and thus produce a fatal septicemia. Living pneumococci were not demonstrated in any of the 9 mice.

In 2 mice (Nos. 79 and 1457) pneumococci were demonstrated; these experiments will be mentioned in detail below. In all the remaining 531 mice no living pneumococci were demonstrated. The mice, which died in less than 14 days after the subcutaneous injection succumbed to an ordinary infection or to the toxic effect of the injected vaccine.

In the transformation experiments which included mouse 79, two mice were given an injection of vaccine 2, while 4 mice were inoculated with the same dose of vaccine together with colon bacilli, 4 mice together with staph. aureus culture, and 2 mice together with pneumococcus serum Type 14. The vaccine was

made of pneumococci grown in serum broth (heating to 60° for 20 min.).

One mouse which received a subcutaneous injection of 0.25 cc. of vaccine 2 (corresponding to 25 cc. of culture) and 0.25 cc of colon culture diluted 1:10, died three days after the injection. In the peritoneal lavage and in the serum broth cultures from the heart and subcutis, which showed a diffuse growth after incubation for 24 hours, the presence of pneumococci was demonstrated, and they showed distinct capsular swelling with Type 2 serum but not with Type 1 serum. Blood agar plates from the heart, subcutis and peritoneum showed growth both of colon bacilli and pneumococci. The Neufeld reaction performed on the pneumococcus colonies proved the organisms to be pneumococci Type 2.

Further, the agglutinative and capsular titers for these pneumococci were determined for pneumococcus serum of Types 1 and 2. The vaccine was prepared by transferring a single colony to serum broth and incubating it till it showed good growth, *i. e.*, about 12 hours and then adding 1 cc. of 5 % formalin to the culture and incubating this mixture for one hour before the titration was carried out, as mentioned in Chapter I, 5. For Type 1 serum the agglutinative and capsular titer was 0, whereas the titers for Type 2 serum were respectively 320 and 80, which means that the pneumococci isolated from the mouse corresponded to the ones injected also with regard to the agglutinative and capsular titer.

The remaining 11 mice which in this experiment were employed for the test for the sterility of the vaccine survived the injection, and on autopsy, performed in the usual way, no pneumococci were demonstrated. Further, all the tests for sterility in vitro showed sterile media (2 flasks with 100 cc. of ox heart broth, 10 tubes of serum broth and 2 10 % blood agar plates). Further, no pneumococci of Type 2 were demonstrated in the transformation experiment itself in the 5 mice which received injection of vaccine 2 and R 1; 2 of the mice died of R septicemia.

The experiment may be taken to indicate that the vaccine contained some living pneumococci and that these were injured

by the heating. On injection of this vaccine alone into mice, the heat-damaged pneumococci were not able to produce a fatal septicemia but were killed by the mouse. If, on the other hand, the resistance of the mouse was lowered at the same time—for instance, by injection of colon bacilli—the pneumococci would be able to multiply, recover their virulence and kill the mouse. Whether this explanation be correct in the present case cannot be decided with certainty. Further, the possibility of contamination or possible technical errors have to be taken into consideration, especially as this experiment was one of my first ones, before the technique was mastered perfectly. This vaccine was prepared of pneumococci grown in serum broth, whereas my later experiments were carried out most often with ox heart broth instead of serum broth, as other experiments—mentioned in Chapter III, 1—had shown that pneumococci grown in serum broth had to be heated to a higher temperature in order to be killed than was required for pneumococci grown in ox heart broth.

As repeated experiments with simultaneous injection of Type 2 vaccine and colon bacilli into mice turned out completely negative, it may safely be taken for granted that the Type 2 vaccine heated to 60° is sterile in practically every case.

In the transformation experiment which included mouse 1457, two mice were given a subcutaneous injection of 0.75 cc. of vaccine 2 (60° for 20 min.) made of Type 2 pneumococci that were sensitive to M & B. Both mice survived the injection and were killed and autopsied in the usual manner 16 days after the injection. In one (mouse 1457) the blood agar plate inoculated from the peritoneum showed two pneumococcus colonies which gave capsular swelling with Type 2 serum and presented the same sensitivity to M & B as did the vaccine employed. In the other mouse and the 20 mice which were inoculated with vaccine 2 together with R 1, no Type 2 pneumococci were demonstrated; nor were any living Type 2 demonstrated in the sterility tests in vitro.

No doubt the presence of these pneumococci of Type 2 is contributable to contamination, just as in the case reported by *Griffith* (38). This view finds support in the following circum-

stances: 1) The two colonies were located at the wall of the dish, outside the streak of inoculation on the plate. 2) No capsulated pneumococci were found in the peritoneal lavage, and 3 blood agar plates inoculated with the same loop from the peritoneal lavage showed no growth of pneumococci. 3) The mouse was perfectly well when it was killed, and no growth of pneumococci was obtained in cultures from the heart's blood and subcutis.

All my experiments in vivo were carried out on mice. In order to make sure that these test mice did not harbor pneumococci, in 20 mice the nasal mucosa was examined in the same way as employed by *Neufeld & Etinger-Tulczynska* (64), by lavage of the nasal cavity, though not with sterile water but with serum broth. With a Record syringe the broth was squirted through a fine needle into one nostril, and it ran out through the other nostril and through the mouth, where it was collected in a sterile test-tube. Furthermore, swabs were taken from the throat of the 20 mice and placed in another tube with serum broth. Both tubes were incubated at 37° for 24 hours and then examined for pneumococci. From both tubes a loopful of serum broth was transferred to 10 % blood agar plates which were incubated for 24 hours and then examined for growth of capsulated pneumococci. Pneumococci were not isolated from any of the mice. Nine days after, this examination was repeated on the same 20 mice exactly as before, and this time too, no growth of pneumococci was obtained.

Chapter IV.

TRANSFORMATION EXPERIMENTS IN VIVO

The present experiments were carried out on white mice with the technique given by *Griffith* (38). A small amount of R pneumococci of one type was injected subcutaneously in the left groin together with a larger amount of heat-killed culture of the S form of another type. The R culture was a 6-8-hour serum broth culture. The vaccine was prepared by heating high-virulent capsulated pneumococci to 60° for 20 min.; as a rule, these pneumococci had been grown in ox heart broth, a few times in serum broth. The S pneumococci were made high virulent by repeated passages through mice, and before the production of each batch of vaccine 1, 2 and 8, a complete virulence test was carried out (Chapter I, 3). When the mice died, autopsy was performed and cultures in serum broth and on 10 % blood agar plate were made from the subcutis, heart and peritoneal cavity. These cultures were incubated for 24 hours at 37°, and then examined with the Neufeld reaction for capsulate pneumococci with sera of the two S types from which the R pneumococci and the vaccine originated.

Transformation experiments were made from Type 1 to Types 2, 6, 8, 14, 20, 22 and 35, from Type 2 to Type 1, and from Type 8 to Type 1.

1. *Transformation of Type after Griffith.*

Experiments on Transformation of Type 1 to Type 2.

The first experiment was carried out with an R 1 strain obtained from S 1 after 26 passages in homologous immune serum. Type 2 was grown in serum broth, and the vaccine was prepared from the centrifugate after heating to 60° for 20 min. The sterility of the vaccine was tested by transferring loopfuls of it to various culture media and observing them for 8 days, also by inoculation of two mice with 0.25 cc. of the vaccine. The experiment itself was carried out on 5 mice which were given a subcutaneous injection in the left groin of 0.25 cc. of vaccine S 2 and 0.25 cc. of R 1 culture. The outcome is recorded in Table 8.

TABLE 8

Living R pn.	Killed S pn.	No. of mice	Results
	Type 2	2	2 L^{14}
Type 1	Type 2	5	3 L^{14}
			2 $D^{3,7}$ = *Type 2*

R pn. = non-capsulated pneumococci.
S pn. = capsular pneumococci.
2 L^{14} = 2 mice living after 14 days.
2 $D^{3,7}$ = 2 mice dead after 3 and 7 days of pneumococcus sepsis Type 2.

From Table 8 it will be noticed that capsulated pneumococci of the same type as the vaccine were grown from two of the five mice.

The experiment was repeated, but this time with an R 1 strain formed from S 1 after 45 passages in homologous immune serum. Type 2 was grown in ox heart broth, and the vaccine was made from the centrifugate after heating to 60° for 20 min.

The sterility of the vaccine was tested by inoculation of various culture media and observing them for 8 days, and also by subcutaneous inoculation of mice with 0.75 cc.: 4 mice received this dose of vaccine alone, 10 mice received it together

with 0.5 cc. of a colon culture diluted 1:20, and 8 mice received it together with intraperitoneal injection of 0.5 cc. of mucin.

The experiment itself was carried out on 10 mice with subcutaneous inoculation in the left groin of 0.75 cc. of vaccine S 2 and 0.25 cc. of R 1 culture. The outcome is shown in Table 9.

TABLE 9

Living R pn.	Killed S pn.	No. of mice	Results
Type 1		4	4 L^{16}
	Type 2	4	4 L^{16}
Type 1	Type 2	10	5 L^{16}
			5 D^{2-3} = *Type 2*

From Table 9 it will be seen that capsulated pneumococci of the same type as the vaccine were cultivated from 5 of the 10 mice.

The next experiment was carried out with an R 1 strain formed from S 1 after 37 passages in immune serum. Type 2 was grown in ox heart broth. The vaccine was prepared from the centrifugate by heating to 60° for 20 min., partly in thin-walled glass ampullas of 5 cc., partly in thick-walled sealed test-tubes. The sterility of the vaccine was examined by inoculating various culture media with it and observing them for 8 days, partly by subcutaneous injection of 0.5 cc. into 2 mice, by injection of the same dose together with 0.5 cc. of a colon culture diluted 1:10 into 10 mice and by injection of this dose simultaneously with intraperitoneal injection of 0.25 cc. of mucin into 10 mice.

The experiment itself was carried out on 10 mice which were given a subcutaneous injection in the left groin of 0.5 cc. of vaccine S 2 and 0.25 cc. of R 1 culture. Five of the mice were inoculated with the vaccine from the ampullas, 5 mice with the vaccine from the test-tube. The results are given in Table 10.

From Table 10 it is seen that capsulated pneumococci of the same type as the vaccine were grown from 6 of the 10 mice. Three of these 6 mice were inoculated with the vaccine from ampullas, the remaining 3 with vaccine from the test-tubes.

TABLE 10

Living R pn.	Killed S pn.	No. of mice	Results
Type 1		4	4 L^{14}
	Type 2	2	2 L^{14}
Type 1	Type 2	10	3 L^{14}
			1 $D^{2} = R$
			6 $D^{2-6} =$ *Type 2*

1 $D^{2} = R = 1$ mouse died after 2 days of R pneumococcus sepsis.

Besides these three experiments, 5 additional experiments on transformation of type were made with altogether 37 mice. In 2 of these experiments the S pneumococci were grown in serum broth, and the remaining 3 in ox heart broth. The vaccine was prepared by heating the centrifugate to 60° for 20 min. The R 1 strain was obtained from S 1 by 30, 34, 41, 50 and 78 passages in about 14 % homologous immune serum. 20 mice were given a subcutaneous injection of 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2; 9 mice received 0.25 cc. of R 1 culture and 0.25 cc. of vaccine S 2; and 8 mice received 0.25 cc. of R 1 culture and 0.5 cc. of vaccine S 2. In none of the 37 mice did the attempt at transformation from Type 1 to Type 2 turn out successful.

Attempts at such transformation with vaccine S 2 prepared by heating to 60° for a shorter or longer period or by heating to a temperature other than 60° were made 9 times with 65 mice altogether. Thus, in 3 experiments the pneumococci were heated for 30 min., once for 15, 40, 50, 60 and 90 min. at 60°, and once to 100° for 60 min. The R 1 strain employed in these experiments was produced from the homologous S 1 strain by 26, 27, 28 (4 times), 29, 33 and 62 passages in immune serum. 45 mice were given a subcutaneous injection of 0.25 cc. of R 1 culture and 0.25 cc. of vaccine S 2, while 10 mice received 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 10 mie 0.1 cc. of R 1 culture and 1.0 cc. of vaccine S 2. But in none of the 65 mice was the attempt at transformation from Type 1 to Type 2 successful.

Experiments on Transformation of Type 1 to Type 35.

The following experiment was carried out with an R 1 strain obtained from S 1 by 53 passages in homologous immune serum. Type 35 was grown in ox heart broth, and the vaccine was made from the centrifugate by heating to 60° for 20 min. The sterility of the vaccine was tested by transfer to various culture media and observe them for 8 days and by inoculating 2 mice subcutaneously with 0.75 cc.

The experiment itself was carried out with 20 mice, 10 of which were given a subcutaneous injection in the left groin of 0.75 cc. of vaccine S 35 and 0.2 cc. of R 1 culture diluted 1 : 7, while 10 received an injection of 0.75 cc. of vaccine S 35, 0.1 cc. of R 1 culture and 0.3 cc. of anti-R 1 serum. The outcome is recorded in Table 11.

TABLE 11

Living R pn.	Killed S pn.	No. of mice	Results
Type 1		2	2 L^{16}
	Type 35	2	2 L^{16}
Type 1	Type 35	20	12 L^{16}
			7 D^{1-3} = R
			1 D^{2} = *Type 35*

Table 11 shows that from one of the 20 mice growth was obtained of capsulated pneumococci of the same type as the vaccine.

In addition 5 more experiments were carried out on a total of 72 mice. The R 1 strain employed in these experiments was obtained from S 1 by 33, 36, 45, 45 and 46 passages in homologous immune serum. 20 mice were injected subcutaneously in the left groin with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 35, 20 mice with 0.25 cc. of R 1 culture and 0.25 cc. of vaccine S 35, 20 mice with 0.25 cc. of R 1 culture and 0.1 cc. of vaccine S 35, and 12 mice with 0.15 cc. of R 1 culture and 0.75 cc. of vaccine S 35. But in none of the 72 mice did the attempt at transformation of Type 1 to Type 35 turn out successful.

A complete virulence test—as mentioned in Chapter I, 3—was now carried out with Types 1, 35 and the new-formed Type 35, eight dilutions being made of a 12-hour serum broth culture. The tubes were designated as 1, 2, 3 ..... 8 and contained the pneumococcus culture respectively in dilutions 1 : 20, 1 : 200, 1 : 2000 . . . . . 1 : 200000000. From each tube, 0.2 cc. was injected intraperitoneally into 2 mice, and when the mice died, a loopful of heart's blood was spread on blood agar plate and transferred to serum broth. After incubation of these cultures at 37° for 24 hours, the pneumococci were examined for capsular swelling with serum of the respective type. Furthermore, 0.2 cc. from each of tubes 5, 6, 7 and 8 was transferred to 10 % blood agar plates which were incubated for 24 hours, and then the pneumococcus colonies were counted.

0.2 cc. of Type 1 culture in dilution 1 : 200000000 contained 1 pneumococcus, and both the injected mice died of pneumococcus sepsis Type 1. 0.2 cc. of Type 35 culture from tubes 7 and 8 killed none of the two mice, whereas 0.2 cc. of dilution 1 : 2000000 killed one of the two mice. 0.2 cc. of this dilution contained 70 pneumococci.

0.2 cc. of the transformed Type 1 culture in tubes 6, 7 and 8 killed none of the mice, whereas both mice were killed by injection of 0.2 cc. from tubes 5 (dilution 1 : 200000). 0.2 cc from tubes 5 and 6 contained respectively 502 and 47 pneumococci.

This virulence test shows that the transformed Type 1 possesses the same virulence as vaccine Type 35.

Experiments on Transformation of Type 1 to Types 8, 14, 20 and 22.

These experiments were carried out respectively 1, 2, 2 and 5 times on a total of 147 mice. The R 1 strain here employed was obtained from S 1 by 5 passages (experiments with vaccines 14 and 20), 32 passages (experiment with vaccine 8), 42, 50, 64, 101 and 104 passages (experiments with vaccine 22) in homologous immune serum. 34 mice were injected subcutaneously with 0.1 cc. of R 1 culture and 0.75 cc. of vaccine, 24 mice with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine, 22 mice with 0.25 cc. of R 1 culture and 0.25 cc. of vaccine, 20 mice with

0.25 cc. of R 1 culture and 0.2 cc. of vaccine, 20 mice with 0.25 cc. of R 1 culture and 0.25 cc. of vaccine, 10 mice with 0.25 cc. of R 1 culture and 0.5 cc. of vaccine, 10 mice with 0.1 cc. of R 1 culture and 0.5 cc. of vaccine, and 7 mice with 0.1 cc. of R 1 culture and 0.25 cc. of vaccine. But in none of 147 mice did the attempt at transformation turn out successful.

2. *Experiments with M&B-sensitive and M&B-resistant Pneumococcus Strains.*

The experiments in the next two sections were aimed to show whether the body of the new-formed pneumococcus were the same as that of the injected R strain. The experiments were carried out with "marked" strains, *i. e.*, strains with demonstrated constant properties connected with the body—as, for instance, chemoresistance and fermentation power. If the mice experiments were performed with an R form with a chemoresistance or fermentation power different from that of the S form, it would be possible to decide from which of the two forms the body of the new-formed pneumococcus originated.

The chemoresistance of the pneumococci was determined for sulfapyridine (M&B) with employment of the technique given by *Schmith* (79) (see Chapter I, 9). From 8 dilutions of M&B in serum broth (1 : 5000, 1 : 10000, 1 : 20000 . . . . . 1 : 640000) 5 cc. were pipetted off into a series of 8 test-tubes, which with the decreasing M&B concentrations were numbered from 1 to 8, so that tube 1 contained the strongest M&B solution, tube 8 the weakest. When the sensitiveness of a pneumococcus to M&B was to be determined, 0.05 cc. of a 24-hour culture diluted 1 : 2000 was added to a series of M&B tubes, which then were incubated at 37° for 24 hours. Then it was noted which tubes had become opaque from growth, and which tubes remained clear because of the bacteriostatic or bactericidal power of the M&B solution. After additional incubation for 24 hours, it was again noted which tubes remained clear, and which had become opaque.

By numbering the tubes in each series from 1 to 8 the sensitiveness of the pneumococci could be expressed by the numbers of the tubes that remained clear after 24 and 48 hours, respec-

tively. Thus, sensitiveness 6 : 4 would mean that tube 6 with the M & B concentration of 1 : 160000 was clear 24 hours after the addition of pneumococcus culture, and that tube 4 with the M & B concentration of 1 : 40000 was clear 48 hours after the addition of the culture.

Further, these experiments were carried out with pneumococcus strains which originally had been sensitive to M & B but were made resistant in this way: a series of M & B tubes were inoculated with the sensitive pneumococcus strain, and after incubation for 48 hours a new series of tubes were inoculated with the first opaque tube from the preceding series. When this procedure was repeated several times, the pneumococci gradually became resistant to M & B.

The chemoresistance of the pneumococci was not affected by storage, mouse passages or culture passages in serum broth. Both *Schmith* and I have examined many pneumococcus strains after intervals of up to 2 years during which they had been stored at room temperature as dried serum broth culture or in ice-box (4°) in Truche with an addition of gelatine, or after the strains had gone through numerous mouse passages and culture passages in serum broth. In no instance did a resistant strain turn sensitive or vice versa. The sensitiveness might vary 1 or 2 tubes when the determination took place in two different batches of M & B dilutions; but this was not due to a change in the sensitiveness of the pneumococci to M & B—on the contrary, to a change in the quality of the serum broth in the two batches or to inaccuracy in the preparation of the M & B solutions. So the chemoresistance of a pneumococcus is a constant property.

The R form of the pneumococcus strain proved to possess the same resistance as the S form from which it originated.

The following experiments were carried out with the R and S forms of resistant and sensitive strains of Types 1, 2 and 8. The resistant forms of these types were obtained from the sensitive strain after respectively 13, 10 and 9 serial cultivations in M & B. Prior to each experiment the sensitivity was determined for both the R and the S pneumococci.

Experiments on Transformation of Type 1 to Type 2 (resistant R 1 + sensitive vaccine S 2).

The M & B sensitivity of the R 1 and S 2 strains employed for these experiments was respectively 2:2 and 6:4. The vaccine was prepared by heating to 60° for 20 min. of the centrifugate from a Type 2 culture in ox heart broth. Altogether 10 experiments were carried out with 20 mice in each. The first 7 experiments were performed with an R 1 strain obtained from S 1 by 62 passages in homologous immune serum. In none of the 140 mice was Type 1 transformed to Type 2. Of these 140 mice, 125 were inoculated subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, 10 mice with 0.1 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 5 mice with 0.1 cc of R 1 culture and 1.0 cc. of vaccine S 2. In addition, 20 of the mice were injected intraperitoneally with 1 cc. of 10 % alcohol 30-60 min. before the injection of R 1 and vaccine S 2.

On employment of an R strain obtained from the homologous S 1 by more than 25 passages in immune serum—as seen from these seven experiments and from the eight experiments in the preceding section—the transformation turned out successful only in 3 experiments (20 %), and at that, only in 13 out of the 202 mice (6.4 %) treated in this way.

The last 3 experiments (Tables 12, 13 and 14) were carried out on altogether 60 mice with an R strain formed from S 1 after only 7 passages in homologous immune serum.

TABLE 12

Living R pn. res.	Killed S pn. sens.	No of mice	Results
Type 1		2	2 L^{14}
	Type 2	4	4 L^{14}
Type 1	Type 2	20	16 L^{15}
			1 $D^{5} = R$
			3 $D^{3-5} =$ *Type 2 res.*

R pn. res. = non-capsulated pneumococci resistant to M & B.
S pn. sens. = capsular pneumococci sensitive to M & B.

15 mice were inoculated subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 5 mice with 0.1 cc. of R 1 culture and 1.0 cc. of vaccine S 2. In addition, 10 of the mice were injected intraperitoneally with 1.0 cc. of 10 % alcohol 30 min. before the injection of vaccine S 2 and R 1. The sterility of the vaccine was tested by injecting 2 mice subcutaneously with 0.75 cc. of vaccine S 2, and 2 mice with 1.0 cc. (in addition, 2 of these mice were given an intraperitoneal injection of 10 % alcohol) and by inoculating several of the aforementioned culture media with the vaccine. All the mice were autopsied and examined in the usual manner, and 3 of the 20 mice yielded growth of capsulated pneumococci of Type 2. One of these three mice had received an injection of alcohol and in this case the new-formed Type 2 could not be isolated as all the blood agar plates and serum broth cultures were contaminated with proteus bacilli. 16 mice survived; 1 mouse died of R pneumococcal sepsis.

The new-formed Type 2 colonies as a rule were large, smooth, greyish in color, looking a little greasy and resembling completely the colonies from the injected Type 2; but some colonies were small and did not present the smooth greasy surface. The latter colonies could not be distinguished morphologically from the R colonies. With Type 2 serum, as a rule, the new-formed pneumococci showed a distinct capsular swelling resembling that of the ordinary Type 2 pneumococcus, but in some of the new-formed pneumococci the capsular swelling was less distinct and appeared only after standing for 30-60 min. with immune serum. On examination of the new-formed pneumococci from the same mouse, the capsule in some of them was found to be thickened but slightly, in others faintly, and in still others it was fully developed. Furthermore, in a few of these pneumococci the capsular swelling was uneven as if it had some »bumps«.

1 cc. of a 12-hour serum broth culture of the new-formed pneumococcus with poor capsular swelling was injected intraperitoneally into mice, which were killed within 12 hours. Cultures from these mice showed growth of Type 2 pneumococci which gave a more distinct capsular swelling with Type 2 serum than did the injected Type 2 pneumococci. By repeated mouse

passages the capsular swelling improved, though without becoming so distinct as that of the normal Type 2 pneumococci.

A complete virulence test was carried out with Types 1, 2 and new-formed Type 2. All 3 types were high-virulent as 0.2 cc. of dilutions 1 : 200000000 injected intraperitoneally killed the mice within 2-3 days. The new-formed Type 2 pneumococci presented all the properties of pneumococci. They were lanceolate diplococci, bile-soluble, Gram-positive, and did not grow on optochin blood agar.

3 single colonies from the 2 mice in which the transformation was successful were transferred to serum broth, and their sensitivity to M & B was determined. One culture showed sensitivity 3:2, the other two 2:2, *i. e.*, the sensitiveness of the new-formed Type 2 corresponded to that of the injected R 1 (2:2), not to that of vaccine Type 2 (6:4).

The experiment was repeated (Table 13) with employment of the same resistant R 1 strain, obtained from the resistant S 1 by 7 passages in homologous immune serum, and the same S 2 strain for vaccine.

TABLE 13.

Living R pn. res	Killed S pn. sens.	No. of mice	Results
Type 1		2	2 L^{16}
	Type 2	2	2 L^{16}
Type 1	Type 2	20	3 L^{16}
			8 $D^{1-4} = R$
			9 $D^{2-5} =$ *Type 2 res.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 10 mice with 0.25 cc. of R 1 culture and 1.0 cc. of vaccine S 2. The sterility of the vaccine was tested by subcutaneous injection into two mice and cultivation from these mice in several of the aforementioned culture media. All the mice were autopsied and examined in the usual manner. The transformation of Type 1 to Type 2 turned out successful in 9 out of the 20 mice. 8 mice died of R pneumococcal sepsis, while 3 survived.

In this experiment too, the colonies of the new-formed pneumococci on blood agar plates consisted *both* in large, smooth and greyish colonies, which completely resembled those of the injected Type 2 pneumococci and gave distinct capsular swelling with Type 2 serum, *and* small colonies, difficult to distinguish from the R colonies and giving poor capsular swelling with Type 2 sera.

From the blood agar plates inoculated from the 9 mice in which the transformation succeeded, single colonies were transferred to serum broth, and after incubation at 37° for 12 hours the sensitivity of the culture to M & B was determined. Altogether 11 cultures were examined, as 3 colonies were taken from one mouse in order to see whether all the new-formed Type 2 pneumococci were equally resistant, and 1 colony from each of the remaining 8 mice.

4 of the cultures showed sensitivity 2:2, while 6 cultures showed 3:2, and 1 culture 3:3. All the three colonies taken from one mouse presented the same sensitivity. The sensitiveness of the new-formed Type 2 pneumococci corresponded to that of the injected R 1 pneumococci (2:2), not to that of the vaccine Type S 2 (6:4).

The experiment was repeated with the same resistant R 1 strain and the sensitive S 2 strain. The result is recorded in Table 14.

TABLE 14

Living R pn. res.	Killed S pn. sens.	No. of mice	Results
Type 1		2	2 L^{14}
	Type 2	2	2 L^{14}
Type 1	Type 2	20	9 L^{14}
			5 D^{3-7} = R
			6 D^{3-5} = *Type 2 res.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 10 mice with 0.1 cc. of R 1 culture and 1.0 cc. of vaccine S 2. The sterility of the vac-

cine was tested by subcutaneous injection into two mice and cultivation in several of the above-mentioned media. All the mice were autopsied and examined in the usual manner. The transformation of Type 1 to Type 2 succeeded in 6 of the 20 mice. 5 mice died of R pneumococcal sepsis, while 9 mice survived.

The new-formed pneumococcal colonies on the blood agar plates from all 6 mice resembled those of the injected Type 2 pneumococci, but their capsular swelling with Type 2 serum was hardly so pronounced.

One of the new-formed pneumococcus colonies from each of the 6 mice was transferred to serum broth, and after incubation at 37° for 12 hours the sensitiveness to M & B was determined for the 6 cultures. Three cultures showed sensitivity 2:2, while 2 cultures showed 3:2 and 1 culture 4:3, *i. e.*, the same sensitiveness as the injected R 1 pneumococci (2:2).

In the last three experiments (cf. Tables 12, 13 and 14) with employment of an R 1 strain formed from S 1 after only 7 passages in homologous immune serum, transformation of Type 1 to Type 2 succeeded in all three experiments (100 %)—in 18 (30 %) of the 60 mice treated in this way.

The experiments showed that the new-formed type received its type-specific properties from the capsular substance of the vaccine employed, whereas its species-specific character originated from the R strain. The experiments further show that the attachment of the capsule to the bacterial body took place gradually, the capsular swelling in some of the new-formed pneumococci being poor and distributed unevenly over the body, while in others it was well-developed and resembled completely the capsular swelling of the injected vaccine, and also that the attachment of the capsule took place moore readily when the experiments were carried out with an R strain obtained from the homologous S strain after a few passages in immune serum.

Experiments on Transformation of Type 1 to Type 2 (sensitive R 1 + resistant vaccine S 2).

The possibility seemed not inconceivable that in the heating of the Type 2 culture the sensitiveness of the pneumococci to M & B might be altered so that the sensitive pneumococci became resistant. If this were the case, the experiments with R 1 resistant and vaccine S 2 sensitive would not be able to show whether the bacterial body of the new-formed Type 2 corresponded to the bacterial body of the R form. Hence the following experiments were carried out with R 1 sensitive and vaccine S 2 resistant. If in these experiments too the resistance of the new-formed pneumococcus was found to correspond to the resistance of the injected R pneumococci, a transformation from Type 1 to Type 2 would have taken place, as the process of heating is not likely once to alter the sensitiveness of a culture to M & B to resistance and the next time, under exactly the same experimental conditions, alter the resistance of this pneumococcus culture to sensitiveness.

The R 1 and S 2 strains employed in these experiments possessed an M & B sensitivity of 6:4 and 1:0, respectively. The vaccine was prepared from a 10-12-hour S 2 culture in ox heart broth by heating to 60° for 20 min.

The first three experiments (Tables 15, 16 and 17) were carried out with a sensitive R 1 strain obtained from the homologous S 1 strain by 5 passages in immune serum.

TABLE 15

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	2 L^{15}
	Type 2	2	2 L^{15}
Type 1	Type 2	20	20 D^{2-3} = *Type 2 sens.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 10 mice with 0.25 cc. of R 1 culture and 1.0 cc. of vaccine S 2. The sterility of the vaccine was tested by subcutaneous injection into 2 mice and by

subcutaneous injection of the vaccine together with colon bacilli into 5 mice, besides by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner and transformation of Type 1 to Type 2 succeeded in all 20 mice.

On blood agar plate, in most instances, the new-formed pneumococcus colonies from all 20 mice resembled the injected pneumococci of Type 2, and they gave a large capsular swelling with Type 2 serum.

One of the new-formed pneumococcus colonies from each of the 20 mice was transferred to serum broth, and after incubation at 37° for 12 hours the M & B sensitivity of the 20 cultures was determined. 1 culture showed sensitivity 6:4, 3 cultures 6:5, 8 cultures 5:5, and 8 cultures 5:4, *i. e.*, all the new-formed pneumococci possessed the same resistance to M & B as the injected R 1 pneumococci (6:4), not as the vaccine Type S 2 (1:0).

The experiment was repeated with the same sensitive R 1 strain and resistant S 2 strain (Table 16).

TABLE 16

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	1 L^{14}, 1 D^{6}
	Type 2	2	1 L^{14}, 1 D^{6}
Type 1	Type 2	20	20 D^{2-3} = *Type 2 sens.*

1 D^{6} = 1 mice died after 6 days of an ordinary intercurrent infection.

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 10 mice with 0.1 cc. of R 1 culture and 1.0 cc. of vaccine S 2. The sterility of the vaccine was tested by subcutaneous injection on 2 mice, and by such injection together with colon bacilli on 5 mice, besides by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner. In all 20 mice the transformation from Type 1 to Type 2 was successful.

On blood agar plate the new-formed pneumococcus colonies were hardly as large and greasy as those of the injected Type 2

pneumococci, and they gave a smaller capsular swelling with Type 2 serum.

One of the new-formed pneumococcus colonies from each of the 20 mice was transferred to serum broth and after incubation at 37° for 12 hours, the sensitivity of the 20 cultures for M & B was determined. 13 cultures showed a sensitivity of 5:5, 3 cultures showed 5:4, and 4 cultures 4:4, *i. e.*, all the new-formed pneumococci possessed the same resistance to M & B as the injected R 1 pneumococci (6:4), not as the vaccine Type S 2 (1:0).

The experiment was repeated with the same sensitive R 1 strain and resistant S 2 strain (Table 17).

TABLE 17

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	2 L^{14}
	Type 2	2	2 L^{14}
Type 1	Type 2	20	1 L^{14}
			1 $D^{2} = R$
			18 $D^{2 \cdot 5} =$ *Type 2 sens.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 10 mice with 0.1 cc. of R 1 culture and 0.75 cc. of vaccine S 2. The sterility of the vaccine was tested by subcutaneous injection of the vaccine on 2 mice and by injection of the vaccine together with colon bacilli on 5 mice, besides by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner. In 18 of the 20 mice the transformation from Type 1 to Type 2 was successful. 1 mouse died of R pneumococcal sepsis, and 1 mouse survived.

As in the two preceding experiments, the cultures showed, besides the typical Type 2 pneumococci, also new-formed pneumococci which were transitional forms between R and S 2 pneumococci.

From each of the 18 mice a single colony was transferred to serum broth, and after incubation at 37° for 12 hours the sen-

sitivity of the 18 cultures to M & B was determined. 1 culture showed sensitivity 6:4, 4 cultures 6:5, 9 cultures 5:5, 3 cultures 5:4 and 1 culture 4:4, *i. e.*, all the new-formed pneumococci possessed the same resistance to M & B as the R 1 pneumococci (6:4), not as the vaccine Type S 2 (1:0).

The experiment was repeated but this time with a sensitive R 1 strain obtained from the homologous S 1 strain by two passages in immune serum and the resistant S 2 strain (Table 18).

TABLE 18

Living R pn sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	2 L^{14}
	Type 2	2	2 L^{14}
Type 1	Type 2	10	4 D^{8-12}
			4 $D^{2-8} = R$
			2 $D^{3-4} =$ *Type 2 sens.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2. The sterility of the vaccine was tested by subcutaneous injection of the vaccine on 2 mice and by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner. In 2 out of the 10 mice the transformation from Type 1 to Type 2 turned out successful. 4 mice died of R pneumococcal sepsis, 4 mice of an ordinary intercurrent infection.

The colonies of the new-formed pneumococci on blood agar plate and their capsular swelling with Type 2 serum resemble precisely the features of the injected Type 2 pneumococci.

One colony of the new-formed pneumococci from each mouse was transferred to serum broth, and after incubation at 37° for 12 hours the sensitivity of the colonies to M & B was determined. Both cultures showed a sensitivity of 6:5, *i. e.*, both the new-formed pneumococci possessed the same resistance to M & B as the injected R 1 pneumococci (6:4), not as the vaccine Type S 2 (1:0).

The experiment was repeated with the same sensitive R 1 strain obtained from S 1 by two passages in homologous immune serum and the same resistant S 2 strain.

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, but transformation from Type 1 to Type 2 failed in all the mice.

The next experiment was carried out with a sensitive R 1 strain obtained from S 1 by three passages in homologous immune serum and the resistant S 2 strain (Table 19).

TABLE 19

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	2 L^{19}
	Type 2	2	2 L^{19}
Type 1	Type 2	10	8 L^{19}
			2 D^{4-5} = *Type 2 sens.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2. The sterility of the vaccine was tested by subcutaneous injection of the vaccine on 2 mice and by cultivation in several of the aforementioned media. In 2 out of the 10 mice the transformation of Type 1 to Type 2 was successful. 8 mice survived.

The new-formed pneumococcus colonies on blood agar plate resembled completely those of the injected Type 2 pneumococci, and gave a large and distinct capsular swelling with Type 2 serum.

From both of the mice in which the transformation was successful, one colony was transferred to serum broth and incubated at 37° for 12 hours. Then the sensitivity of both cultures to M & B was determined. One culture showed sensitivity 6:5, the other culture 5:5, *i. e.*, the new-formed pneumococci possessed the same resistance to M & B as the injected R 1 pneumococci (6:4), not as the vaccine Type S 2 (1:0).

The following experiment was carried out with a sensitive R 1 strain obtained from the homologous S 1 strain by 4 pas-

sages in immune serum and the resistant S 2 strain. 10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2. The attempt at transformation from Type 1 to Type 2 failed in all 10 mice.

From the experiments on transformation from Type 1 to Type 2 with R 1 sensitive and vaccine S 2 resistant, it will be noticed that on employment of an R 1 strain obtained from S 1 by up to 5 passages in immune serum, transformation from Type 1 to Type 2 succeeded in 5 out of 7 experiments (71.4 %)—in 62 (62 %) of the 100 mice included in the experiments. It is further seen that the new-formed pneumococci acquired the type-specific properties from the capsular substance of the vaccine employed, while the species-specific character originated from the R strain.

The last two series of experiments showed that the transformation of one specific type to another specific type was genuine and not ascribable to surviving pneumococci in the vaccine, as the new-formed pneumococci had acquired the same properties as were possessed by the injected R pneumococci, and that the transformation was due to the attachment of a capsule from the capsular substance of the vaccine on the injected R pneumococcus.

Experiments on Transformation of Type 2 to Type 1 (R 2 sensitive + vaccine S 1 resistant).

The experiments were carried out with a sensitive R 2 strain and a resistant S 1 strain with a sensitivity to M & B of 5:4 and 2:1, respectively. The vaccine was made of the centrifugate from a 10-12-hour ox heart broth culture by heating to 60° for 20 min.

For the first experiment a sensitive R 2 strain was employed that was obtained from the homologous S 2 by 5 passages in immune serum (Table 20).

10 mice were injected subcutaneously with 0.25 cc. of R 2 culture and 0.75 cc. of vaccine S 1, and 10 mice with 0.25 cc. of R 2 culture and 1.0 cc. of vaccine S 1. The sterility of the vaccine was tested by subcutaneous injection on 2 mice and by in-

TABLE 20

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 2		2	2 L^{14}
	Type 1	2	2 L^{14}
Type 2	Type 1	20	2 L^{14}
			9 D^{2-3} = R
			3 D^{2-7} = Type 2
			5 D^{2-4} = *Type 1 sens.*
			1 D^{3} = *Type 1 sens.* + *Type 2*

1 D^3 = 1 mouse died after 3 days of pneumococcus sepsis Type 1 and Type 2.

jection together with colon bacilli on 5 mice, besides by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner. In 6 of the 20 mice the transformation from Type 2 to Type 1 turned out successful. 3 of the 20 mice died of pneumococcus sepsis Type 2, 9 died of R pneumococcal sepsis, and 2 survived.

On blood agar plates the colonies of the new-formed pneumococci resembled those of Type 1 pneumococci, but their capsular swelling with Type 1 serum varied in size, some of the new-formed pneumococci showing only a slight thickening of the capsule, while others gave a distinct capsular swelling. 0.25-0.5 cc. of a 12-hour serum broth culture of the new-formed pneumococci injected intraperitoneally into mice killed these animals within 48 hours.

From one of the mice that died 3 days after the subcutaneous injection of R 2 culture and vaccine S 1, growth was obtained both of R, S 1 and S 2 pneumococci, *i. e.*, in this mouse, some of the injected R 2 pneumococci had acquired their homologous capsule, and others the heterologous capsule, whereas some got neither the homologous nor the heterologous capsule but remained R pneumococci.

10 colonies of the new-formed pneumococci from the 6 mice were transformed to serum broth, and the sensitivity of the cul-

tures to M & B after incubation for 12 hours was examined. Of these 10 colonies 5 were taken from one mouse in order to see whether the new-formed pneumococci isolated from this animal would show any difference in sensitiveness to M & B. 5 cultures showed a sensitivity of 5:5, 5 cultures showed 5:4, *i. e.*, all the new-formed pneumococci possessed the same resistance as the injected R 2 pneumococci (5:4), not as the vaccine Type S 1 (2:1).

Then 4 experiments were carried out on a total of 78 mice with a sensitive R 2 strain, obtained from the homologous S 2 by 11 and 19 passages in immune serum; 3 of the experiments (58 mice altogether) were performed with R 2 obtained after 11 passages, one experiment (20 mice) with R 2 obtained after 19 passages. 40 mice were injected subcutaneously with 0.25 cc. of R 2 culture and 0.75 cc. of vaccine S 1, 28 mice with 0.1 cc. of R 2 culture and 1.0 cc. of vaccine S 1, and 10 mice with 0.25 cc. of R 2 culture and 1.0 cc. of vaccine S 1. In none of the 78 mice did the attempt at transformation from Type 2 to Type 1 turn out successful.

From these experiments it will be noticed that the transformation of a pneumococcus type to another type took place more easily on employment of an unstable R pneumococcus than on employment of a stable R pneumococcus. For the transformation was accomplished in 30 % of the mice inoculated with R 2 that was obtained from S 2 by only 5 passages in immune serum, whereas it failed in the mice inoculated with R 2 obtained from S 2 by 11 and 19 passages in immune serum.

Experiments on Transformation of Type 2 to Type 1 (R 2 resistant + vaccine S 1 sensitive).

These experiments were carried out with a resistant R 2 strain and a sensitive S 1 strain with a sensitivity to M & B of 0:0 and 5:5, respectively. The vaccine was prepared from the centrifugate from a 10-12-hour culture in ox heart broth by heating to 60° for 20 min.

For the first experiment a resistant R 2 strain was employed that was obtained from the homologous S 2 strain by 8 passages in immune serum (Table 21).

TABLE 21

Living R pn. res.	Killed S pn. sens.	No. of mice	Results
Type 2		2	2 L^{15}
	Type 1	2	2 L^{15}
Type 2	Type 1	20	9 L^{15}
			4 D^{2-3} = R
			1 D^{4} = *Type 2 res.*
			3 D^{3-4} = *Type 1 res.*
			3 D^{3} = *Type 1 res* + *Type 2 res.*

10 mice were injected subcutaneously with 0.25 cc. of R 2 culture and 0.75 cc. of vaccine S 1, and 10 mice with 0.25 cc. of R 2 culture and 1.0 cc. of vaccine S 1. The sterility of the vaccine was tested by subcutaneous injection on 2 mice, and by injection together with colon bacilli on 5 mice, besides by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner. In 6 of the 20 mice the transformation of Type 2 to Type 1 turned out successful. 4 of the 20 mice died of R pneumococcal sepsis, 1 died of pneumococcal sepsis Type 2, and 9 survived.

On blood agar plate the colonies of the new-formed pneumococci were somewhat smaller than the colonies of Type 1, and also more difficult to distinguish from the R colonies; with Type 1 serum, most of them gave a distinct capsular swelling, but a few of the new-formed pneumococci showed capsular swelling only after standing for some length of time, and here the swelling was less distinct. All transitions were seen from a mere thickening of the capsule to distinct capsular swelling. 0.5 cc. of a 24-hour serum broth culture of the new-formed pneumococci injected intraperitoneally into mice killed these animals within 48 hours.

ous injection of R 2 culture and vaccine S 1 showed growth of R, S 1 and S 2 pneumococci, *i. e.*, in these 3 mice some of the injected R 2 pneumococci had recovered their homologous capsule and others had acquired the heterologous capsules, whereas some got neither the homologous capsule nor the heterologous and thus remained R.

Single colonies of the new-formed pneumococci from the 6 mice in which the transformation succeeded were transferred to serum broth and after incubation at 37° for 12 hours the sensitiveness of the cultures to M & B was examined. All 6 cultures showed a sensitivity of 0:0, *i. e.*, the new-formed pneumococci possessed the same resistance as the injected R 2 pneumococci (0:0), not as the vaccine Type S 1 (5:5).

Also the sensitiveness of some serum broth cultures of Type 2 isolated from these mice was determined, and every time their sensitivity was found to be 0:0, *i. e.*, the same sensitivity as that of the injected R 2 pneumococci. Thus the sensitivity of the R 2 pneumococci to M & B was not altered by the subcutaneous injection into mice or by reversion of the organism to the S form.

The experiment was repeated with the same resistant R 2 strain obtained from the homologous S 2 by 8 passages in immune serum and with the same sensitive S 1 strain. 10 mice were injected subcutaneously with 0.25 cc. of R 2 culture and 0.75 cc. of vaccine S 1, and 9 mice with 0.1 cc. of R 2 culture and 1.0 cc. of vaccine S 1. But the attempt at transformation of Type 2 to Type 1 failed in all 19 mice. 2 of the mice died of pneumococcal sepsis Type 2.

Then an experiment was performed with the resistant R 2 strain obtained from S 2 by only 4 passages in immune serum and with the sensitive S 1 strain. 10 mice were injected subcutaneously with 0.25 cc. of R 2 culture and 0.75 cc. of vaccine S 1, and 10 mice with 0.25 cc. of R 2 culture and 1.0 cc. of vaccine S 1. In this experiment the R 2 strain was too unstable, as all 20 mice died of pneumococcus sepsis Type 2. Here the R 2 pneumococci had acquired the homologous capsule, not the heterologous. Also the two control mice injected subcutaneously

with 0.25 cc. of R 2 culture died after 3 days of pneumococcal sepsis Type 2.

Experiments on Transformation from Type 1 to Type 8 (R 1 sensitive + vaccine S 8 resistant).

These experiments were carried out with the employment of a sensitive R 1 strain and a resistant S 8 strain, the sensitivity of which to M & B was respectively 6:4 and 1:1. The vaccine was prepared from the centrifugate of a 10-12-hour S 8 culture in ox heart broth by heating to 60° for 20 min.

One experiment was performed with a sensitive R 1 strain obtained from the homologous S 1 by 48 passages in immune serum (Table 22).

TABLE 22

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	1 L^{14}, 1 D^{5}
	Type 8	4	1 L^{14}, 3 D^{1-2}
Type 1	Type 8	10	9 $D^{1-4} = R$
			1 $D^{3} =$ *Type 8 sens.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 8. The sterility of the vaccine was tested by subcutaneous injection on 4 mice and by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual manner. In one of the 10 mice the transformation from Type 1 to Type 8 succeeded; the other 9 mice died of R pneumococcal sepsis.

The appearance of the colonies of the new-formed pneumococci on blood agar plates and their capsular swelling with Type 8 serum resembled completely those features of the ordinary Type 8 pneumococci. The colonies were large and greasy, the capsular swelling pronounced.

One colony of the new-formed pneumococci was transferred to serum broth, and after incubation at 37° for 12 hours the sensitiveness of the culture to M & B was determined. The sensi-

tivity was found to be 6:5, *i. e.*, the new-formed pneumococci possessed the same resistance as the injected R 1 pneumococci (6:4), not as the vaccine Type S 8 (1:1).

The experiment was repeated but with a sensitive R 1 strain obtained from the homologous S 1 by 54 passages in immune serum (Table 23).

TABLE 23

Living R pn. sens.	Killed S pn. res.	No. of mice	Results
Type 1		2	2 L^{14}
	Type 8	2	2 L^{14}
Type 1	Type 8	10	1 L^{14}
			3 $D^{2} = R$
			6 $D^{2-4} =$ *Type 8 sens.*

10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 8. The sterility of the vaccine was tested by subcutaneous injection on 2 mice and by injection together with colon bacilli on 10 mice, besides by cultivation in several of the aforementioned media. All the mice were autopsied and examined in the usual way. In 6 of the 10 mice the transformation of Type 1 to Type 8 turned out successful, while 3 mice died of R pneumococcal sepsis and 1 mouse survived.

The colonies of the new-formed pneumococci on blood agar plate and their capsular swelling with Type 8 serum resembled completely those features of the ordinary Type 8 pneumococci. One colony of the new-formed pneumococci from each of the 6 mice was transferred to serum broth, and after incubation at 37° for 12 hours the sensitiveness of the cultures to M & B was determined. 1 culture showed sensitivity 6:5, 1 culture 5:5, 1 culture 5:3, and 3 cultures 4:4, *i. e.*, the new-formed pneumococci possessed the same resistance as the injected R 1 pneumococci (6:4), not as the vaccine Type S 8 (1:1).

Two other experiments were performed, one with a sensitive R 1 strain obtained from the homologous S 1 strain by 31 passages in immune serum, the other with an R 1 strain obtained

from S 1 by 39 passages in immune serum. 10 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.25 cc. of vaccine S 8, and 10 mice with 0.25 cc. of R 1 culture and 0.5 cc. of vaccine S 8, but the attempt at transformation of Type 1 to Type 8 failed in all the mice.

Of these 4 experiments the transformation succeeded in 2 (50 %)—in 7 of the 40 mice (17.5 %). But the experiments were carried out with a stable R 1 strain obtained from S 1 by more than 30 passages in homologous immune serum.

Also this series of experiments showed that the new-formed type consisted of the species-specific part of the R form and the type-specific part, the capsule, from the S vaccine.

Experiments on Transformation of Type 1 to Type 8 (R 1 resistant + vaccine S 8 sensitive).

In these experiments a resistant R 1 strain was employed and a sensitive S 8 strain, the sensitivity of the two strains being respectively 2:1 and 6:4. The vaccine was prepared from the centrifugate of a 10-12-hour S 8 culture in ox heart broth by heating to 60° for 20 min.

Altogether 17 experiments were carried out on a total of 321 mice. The first 11 experiments were performed with a stable resistant R 1 strain obtained from the homologous S 1 by numerous passages in immune serum (in one experiment 30 passages, in another 49, in a third 57, and in eight experiments 62 passages). 59 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 8, 51 mice with 0.5 cc. of R 1 culture, and 0.75 cc. of vaccine S 8, 32 mice with 0.3 cc. of R 1 culture and 1.0 cc. of vaccine S 8, 20 mice with 0.25 cc. of R 1 culture and 1.0 cc. of vaccine S 1, 10 mice with 0.25 cc. of R 1 culture and 0.5 cc. of vaccine S 8, 10 mice with 0.5 cc. of R 1 culture and 1.0 cc. of vaccine S 8, 10 mice with 0.4 cc. of R 1 culture and 0.75 cc. of vaccine S 8, and 9 mice with 0.3 cc. of R 1 culture and 0.75 cc. of vaccine S 8. But in none of these 201 mice was the transformation of Type 1 to Type 8 successful.

In one of these experiment, on a total of 24 mice, a group of 6 mice was injected subcutaneously with 0.5 cc. of R 1 culture and 0.75 cc. of vaccine S 8. None of these mice died of Type 8 sepsis, but 3 died of R pneumococcal sepsis. Then the next group of 6 mice were injected subcutaneously with vaccine S 8 and an R culture isolated from one of the dead mice. As the attempted transformation of type failed in these 6 mice too, the experiment was continued in two additional groups of 6 mice, each group being injected with vaccine S 8 and an R culture isolated from one of the mice of the preceding group that had died of R pneumococcus sepsis. This experiment was performed in order to see whether the transformation from Type 1 to Type 8 would take place more readily when the experiment was carried out with an R strain which had passed through mice several times. But no transformation from Type 1 to Type 8 took place in any of the 24 mice.

For the last 6 experiments on 120 mice altogether a resistant R 1 strain was employed that was obtained from the homologous S 1 pneumococcus by 7 passages in immune serum. This R 1 strain was the same as had been employed in the experiments on transformation of Type 1 to Type 2, in which the transformation had turned out successfully. 60 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 8, 20 mice with 0.25 cc. of R 1 culture and 1.0 cc. of vaccine S 8, 10 mice with 0.1 cc. of R 1 culture and 0.75 cc. of vaccine S 8, 10 mice with 0.35 cc. of R 1 culture and 1.0 cc. of vaccine S 8, 10 mice with 0.1 cc. of R 1 culture and 1.0 cc. of vaccine S 8, and 10 mice with 0.35 cc. of R 1 culture and 0.75 cc. of vaccine S 8. But in none of the 120 mice was transformation from Type 1 to Type 8 accomplished.

Experiments on Transformation of Type 8 to Type 1 (R 8 resistant + vaccine S 1 sensitive).

The first 4 experiments were carried out with employment of a resistant R 8 strain obtained from strain Type 8 No. 12455 and the sensitive S 1 pneumococcus, with a sensitivity of 2:1 and

6:4, respectively. The vaccine was prepared from the centrifugate of a 10-12-hour culture in ox heart broth by heating to 60° for 20 min.

The experiments, on 60 mice altogether, were performed with a resistant R 8 strain obtained from S 8 by 35, 38, 83 and 83 passages in homologous immune serum. 30 mice were injected subcutaneously with 0.25 cc. of R 8 culture and 0.75 cc. of vaccine S 1, 10 mice with 0.1 cc. of R 8 culture and 0.6 cc. of vaccine S 1, 10 mice with 0.25 cc. of R 8 culture and 1.0 cc. of vaccine S 1, and 10 mice with 0.4 cc. of R 8 culture and 0.75 cc. of vaccine S 1; the last 10 mice were injected simultaneously with 0.1 cc. of anti-R 8 serum. In none of the 60 mice did the attempt at transformation of Type 8 to Type 1 succeed. 39 of the mice died of S 8 pneumococcal sepsis, as the R 8 strain here employed was not stable but reverted to S 8 on injection into mice together with a heterologous vaccine. In the experiments with R 8 obtained from S 8 by 83 passages in immune serum, 29 of the 40 mice injected died of Type 8 pneumococcal sepsis. In the control mice injected with R 8 alone, reversion of R 8 took place only in the strain obtained from S 8 by 35 passages, not in the strains obtained by 38 and 83 passages.

As cultivation of S 8 in homologous immune serum did not result in stable R strains, the next 3 experiments (on 60 mice altogether) were carried out with another resistant R pneumococcus which was obtained from a Type 8 pneumococcus that had been isolated from the sputum of a patient with lobar pneumonia, for it seemed conceivable that the transformation of Type 8 to Type 1 might take place more readily on employment of another Type 8 strain. The S pneumococcus isolated from the sputum was sensitive to M & B (6:5), but after 10 passages in M & B solutions it had become resistant, showing a sensitivity of 1:1.

The resistant R 8 strains employed in these 3 experiments were obtained from S 8 by 7, 8 and 13 passages in homologous immune serum. 30 mice were injected subcutaneously with 0.25 cc. of R 8 culture and 0.75 cc. of vaccine S 1, and 30 mice with 0.25 cc. of R 8 culture and 1.0 cc. of vaccine S 1. The attempt at transformation from Type 8 to Type 1 failed in all 60 mice, and

only 1 mouse died of Type 8 pneumococcal sepsis on employment of R 8 obtained by 7 passages in immune serum. None of the mice injected with R 8 alone died of Type 8 pneumococcal sepsis.

The R pneumococci obtained from the Type 8 isolated from the pneumonic sputum were more stable than the R pneumococci obtained from the Type 8 generally employed (No. 12455), as they were stable already after 8 passages of the homologous S 8 in immune serum, whereas R 8 from strain 8 No. 12455 were not stable even after 83 passages of S 8 No. 12455 in immune serum.

Experiments on Transformation of Type 8 to Type 1 (R 8 sensitive + vaccine S 1 resistant).

These experiments were carried out with a sensitive R 8 strain and a resistant S 1 strain with a sensitivity to M & B of 6:5 and 2:1, respectively. The vaccine was prepared from the centrifugate of a 10-12-hour S 1 culture in ox heart broth by heating to 60° for 20 min.

The first 2 experiments (on 20 mice altogether) were performed with the employment of the R 8 form of strain S 8 No. 12455. In one experiment R 8 was obtained from S 8 by 38 passages in homologous immune serum, in the other by 43 passages. All 20 mice were injected subcutaneously with 0.25 cc. of R 8 culture and 0.75 cc. of vaccine S 1. But in none of the 20 mice was Type 8 transformed to Type 1. Of the 10 mice injected with R 8 obtained from S 8 by 43 passages, 4 died of Type 8 pneumococcus sepsis. The R 8 pneumococci were not stable; on injection into the mice they had recovered their homologous capsule.

The next 5 experiments (on 100 mice altogether) were carried out with the R form of the sensitive Type 8 isolated from the sputum of a pneumonic patient. R 8 was obtained from S 8 by 5, 6, 7, 7 and 13 passages in homologous immune serum. 50 mice were injected subcutaneously with 0.25 cc. of R 8 culture and 0.75 cc. of vaccine S 1, 30 mice with 0.25 cc. of R 8 culture and 1.0 cc. of vaccine S 1, 10 mice with 0.1 cc. of R 8 culture and

1.0 cc. of vaccine S 1, and 10 mice with 0.35 c. of R 8 culture and 0.75 cc. of vaccine S 1. The transformation of Type 8 to Type 1 did not succeed in any of the 100 mice. In the 4 experiments with R 8 obtained from S 8 by 5, 6 and 7 passages in immune serum the R pneumococci were not stable, as 28 of the 80 mice injected with these organisms died of Type 8 pneumococcus sepsis. On the other hand, the R 8 obtained from S 8 by 13 passages in immune serum were stable, as they did not regain their homologous capsule on subcutaneous injection into mice.

3. *Experiments with Strains Differing in Fermentation Power.*

The fermentation experiments were carried out with the employment of Hiss' medium, consisting of 1 part horse serum and 2 parts distilled water. The substances to be fermented were added in an amount of 0.5 %, and bromocresol purple was used for indicator. The medium was poured into narrow test-tubes, to a height of 2 cm.

The experiments were carried out with the following 12 substances: arabinose, xylose, dulcite, inosite, glucose, galactose, lactose, saccharose, inulin, mannite, melibiose and salicin; besides, one tube with Hiss' medium was used for control. To each of the 13 tubes one drop of a 10-12 hour culture of the pneumococcus strain under analysis was added, and the tubes were incubated at 37°; they were inspected daily for 14 consecutive days. If the added substances were fermented by the pneumococci, the acid formation brought about a fall in pH, which originally was 7.8, and the bromocresol purple changed in color from blue to yellow, while the medium coagulated at the same time. In my experiments the substances were reckoned as fermented only when the tubes showed both coagulation and a distinct change in color.

All the pneumococcus strains in the collection of the State Serum Institute were examined in this way—a total of 64 types, with 137 strains, partly Danish strains, partly strains from the Lederle Laboratory in New York. 42 strains were examined twice, a few strains 3 or 4 times. From the tubes where no fer-

mentation took place, numerous subcultures were made on blood agar plate, and growth of pneumococci was obtained every time. In the fermentation tubes the pneumococci kept alive even for 2 weeks after the inoculation of the tube.

These experiments showed that none of the 137 strains fermented l-arabinose, xylose, dulcite and inosite. All the strains fermented glucose, galactose, lactose and saccharose with exception of Type 11, No. 34356, which did not ferment galactose. Galactose was fermented somewhat later than the other three sugars which as a rule were fermented within 3 days.

As to the fermentation of inulin, mannite, melibiose and salicin, the pneumococci differed in their behavior, without any definite aspects being demonstrable as characteristic of the various groups. Thus 85 of the strains (62 %) representing 39 of the types (60.9 %) fermented the polysaccharid inulin, while 25 types did not ferment inulin. The percentage of the strains which fermented inulin is smaller than reported by *Jürgens* (46), as 97.3 % of his 112 strains fermented inulin.

These experiments showed, then, that not all pneumococci are able to ferment inulin, and that fermentation of inulin cannot be used as a differential-diagnostic criterion between pneumococci and other bacteria.

47 strains (34.3 %), representing 19 types (29.7 %) fermented the alcohol mannite, which was fermented chiefly by the higher types. 97 strains (70.8 %) or 53 types (82.8 %) fermented the trisaccharid melibiose; and 126 strains (92 %) or 61 types (95.3 %) fermented the glucoside salicin.

The experiments showed that serologically different types did not differ distinctly with regard to fermentation, and that serologically identical strains did not always behave alike as to fermentation power.

Besides these tests on the fermentation power of the capsulated pneumococci, R pneumococci of Types 1, 2, 3, 8 and 22 were examined 7, 11, 2, 6 and 1 times, respectively, with the same 12 substances, and this experiment showed that R pneumococci essentially ferment the same substances as do the homologous S pneumococci.

The purpose of these fermentation tests was to find 2 pneu-

mococci which differed in their fermentation power. If this difference were constant, it might be utilized to prove that the transformation of one pneumococcus type to another was true. If the new-formed pneumococcus showed the same fermentation power as the injected R pneumococcus, a transformation of type would have taken place; if, on the other hand, the new-formed pneumococcus showed the same fermentation properties as the type of the vaccine, living pneumococci must have been present in the vaccine.

Experiments were therefore carried out to investigate whether the capacity of the pneumococci for the fermentation of the aforementioned 12 substances was constant.

On examination of the 64 types it was found that Types 1, 2, 6 and 8 (strain 8 Henriques) differed distinctly as to fermentation of inulin, mannite, melibiose and salicin. These 4 types and the R form of Types 1 and 2 were then examined once a week for 5 weeks as to the fermentation of these 4 substances, but not of the remaining 8 substances, as they all behaved alike as to the fermentation of these 8 substances.

The experiment was carried out as follows: 0.5 cc. of Truche culture of the 4 S types—1, 2, 6 and 8—were injected intraperitoneally on mice. When the mouse died, cultures from the heart's blood were made in serum broth and on 10 % blood agar plate. After incubation at 37° for 24 hours, 1 drop of the serum broth culture was spread on 10 % blood agar plate, and 1 drop was transferred to Truche medium and to each of 12 tubes with inulin, mannite, melibiose and salicin (3 tubes for each "sugar").

From the R 1 and R 2 cultures, new cultures were made in serum broth without letting the R pneumococci pass through mice; otherwise these organisms were treated in the same manner as the S pneumococci.

The results of the fermentation tests are recorded in Table 24.

From Table 24 it will be noticed there is a distinct difference between R 1 and S 2 as to the fermentation of the sugars here employed and that this difference is constant. Thus R 1 fermented always inulin and never mannite, whereas S 2 never fermented inulin and nearly always mannite. The difference in the fermentation of melibiose and salicin was less distinct and not

TABLE 24

Pneumococcus types	Inulin	Mannite	Melibiose	Salicin
S 1	12 + 3 —	0 + 15 —	1 + 14 —	6 + 9 —
	80 %	0 %	6.7 %	40 %
R 1	15 + 0 —	0 + 15 —	15 + 0 —	6 + 9 —
	100 %	0 %	100 %	40 %
S 2	0 + 15 —	12 + 3 —	15 + 0 —	14 + 1 —
	0 %	80 %	100 %	93.3 %
R 2	3 + 12 —	12 + 3 —	15 + 0 —	14 + 1 —
	20 %	80 %	100 %	93.3 %
S 6	0 + 15 —	4 + 11 —	8 + 7 —	15 + 0 —
	0 %	26.7 %	53.3 %	100 %
S 8	9 + 6 —	0 + 15 —	10 + 5 —	10 + 5 —
	60 %	0 %	66.7 %	66.7 %

S 1 = capsular Type 1 pneumococci.
R 1 = non-capsular Type 1 pneumococci.
12 + = 12 tubes which were fermented in 14 days.
3 — = 3 tubes which were not fermented in 14 days.
% = fermented tubes in per cent of the total number of tubes.

serviceable for differential-diagnostic purposes. Both pneumococci always fermented melibiose, while salicin was fermented more often by S 2 than by R 1.

Then an experiment was carried out to investigate the capacity of 9 new-formed Type 2 pneumococci for fermentation of the aforementioned 12 substances. The results are given in Table 25 but only with regard to the fermentation of inulin, mannite, melibiose and salicin, as all the new-formed types fermented glucose, galactose, lactose and saccharose but not arabinose, xylose, dulcite and inosite.

Table 25 shows that all the transformed types fermented inulin (100 %), but not mannite (100 %), that is, they behaved exactly as the R 1 strain employed, not as the type of the vaccine, S 2. Melibiose and salicin were fermented by the new-

TABLE 25

Pneumococcus Type 2 (1➛2) Mouse No.	Inulin	Mannite	Melibiose	Salicin
11	$+^{1-2}$	—	$+^{8-12}$	$+^{3}$
14	$+^{1-2}$	—	$+^{14}$	$+^{8}$
209	$+^{1-2}$	—	$+^{12}$	—
211	$+^{1-2}$	—	—	$+^{6}$
212	$+^{1-2}$	—	$+^{14}$	$+^{8-10}$
215	$+^{1-2}$	—	—	$+^{14}$
216	$+^{1-2}$	—	$+^{5-8}$	$+^{5-6}$
218	$+^{1-2}$	—	$+^{14}$	$+^{12}$
1457	$+^{2-4}$	—	—	—

+ = fermentation.
— = no fermentation.
Figures = days for commencing and complete fermentation, respectively.

formed type respectively 6 out of 9 times (66.7 %) and 7 out of 9 times (77.8 %).

A new-formed Type 2 pneumococcus was made rough in the usual manner by cultivation in about 14 % homologous immune serum; it was designated as R 2 (1→2). If a transformation of type had taken place, the R form of the new-formed type should show the same fermentation properties towards the 12 sugars as observed for R 1. If, on the other hand, no transformation had taken place, and the Type 2 pneumococci originated from the surviving pneumococci in the vaccine, the R form would have to behave like R 2.

TABLE 26

Pneumococcos Type	Inulin	Mannit	Melibiose	Salicin
R 1	$+^{1-2}$	—	$+^{8}$	—
R 2	—	$+^{4-6}$	$+^{2-4}$	$+^{3-4}$
R 2 (1→2)	$+^{1}$	—	$+^{6-7}$	—

R 2 (1→2) = non-capsulated pneumococcus of the new-formed Type 2 pneumococcus.

The fermentation of the 12 sugars by R 2 (1 → 2) was examined 3 times; arabinose, xylose, dulcite and inosite were not fermented whereas glucose, galactose, lactose and saccharose were fermented each time. The fermentation of inulin, mannite, melibiose and salicin is recorded in Table 26. For comparison, the fermentation of these 4 sugars by R 1 and R 2 is recorded at the same time.

R 2 (1→2) fermented inulin in all three tests (100 %) but not mannite at all (0 %), melibiose in each test (100 %) and salicin in 1 test (33.3 %), *i. e.*, R 2 (1→2) presented exactly the same fermentative qualities as did the R 1 employed for the experiment.

Tables 25 and 26 show plainly that the new-formed type is produced by attachment of the capsular substance of the vaccine employed to the injected R pneumococcus.

Fermentation tests of the 64 types showed that there is no distinct difference between R 1 and Type 8 No. 12455. Still, in one of the 2 tests mannite was fermented by Type 8, whereas R 1 never fermented mannite.

7 of the new-formed Type 8 pneumococci were examined altogether 10 times for fermentation of the 12 sugars, but mannite was not fermented at all.

This experiment also appears to show that a true transformation of Type 1 to Type 8 actually may take place, but nothing definite can be stated about this as the difference in the fermentative properties of R 1 and S 8 is too slight to allow of any conclusion in this respect.

Also the fermentative difference between S 1 and S 35 was slight. In one out of 4 experiments Type 35 fermented mannite —*i. e.*, 25 %—while the new-formed Type 35 was unable to ferment mannite in 11 tests. As to the fermentation of mannite, then, the new-formed type behaved as R 1.

From Table 24 it will be noticed that R 1 and S 6 differed fermentatively, and this difference in fermentation power was constant. Thus, inulin was always fermented by R 1, never by Type 6. The difference was less distinct as to the fermentation of mannite, melibiose and salicin, the three sugars being fermented by R 1 respectively in 0 %, 100 % and 40 % of the tests,

TABLE 27

Living R pn.	Killed S pn.	No. of mice	Results
Type 1		2	2 L^{15}
	Type 6	2	2 L^{15}
Type 1	Type 6	20	18 $D^{1-2} = R$
			2 $D^{3-9} =$ *Type 6*

R pn. = non-capsulated pneumococci.
S pn. = capsular pneumococci.
2 L^{15} = 2 mice living after 15 days.
18 D^{1-2} = 18 mice died after 1-2 days of R pneumococcus sepsis.
2 D^{3-9} = 2 mice died after 3-9 days of pneumococcus sepsis Type 6.

whereas S 6 fermented these sugars in 26.7 %, 53.3 % and 100 % of the tests.

On this account, mouse experiments were carried out with the aim of transforming Type 1 to Type 6. These experiments were made with an R 1 strain obtained from S 1 after 5 passages in homologous immune serum. Vaccine was made of the centrifugate from a 10-12-hour ox heart broth culture of Type 6 by heating to 60° for 20 minutes (Table 27).

10 mice were inoculated subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 6, and 10 mice with 0.25 cc. of R 1 culture and 1.0 cc. of vaccine S 6. The sterility of the vaccine was examined by injecting the vaccine subcutaneously into mice, and together with a culture of colon bacilli into 5 mice; further, by inoculation of several of the aforementioned culture media. All the mice were autopsied and examined in the usual manner. In 2 out of the 20 mice the transformation from Type 1 to Type 6 was successful. The remaining 18 mice died of R pneumococcal sepsis.

The colonies of the new-formed pneumococci on blood agar plate and their capsular swelling with Type 6 serum showed a perfect resemblance to the injected Type 6 pneumococci; no transitional forms were found. Mice inoculated intraperitoneally with 0.5 cc. of a serum broth culture of the new-formed type died within 24 hours.

This experiment was repeated twice with the same R 1 and

S 6 strains. 10 mice were inoculated subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 6, while 10 mice were inoculated with 0.25 cc. of R 1 culture and 0.5 cc. of vaccine S 6, 10 mice with 0.1 cc of R 1 culture and 1.0 cc. of vaccine S 6, and 10 mice with 0.1 cc. of R 1 culture and 0.75 cc. of vaccine S 6. The attempts at transformation of Type 1 to Type 6 succeeded in 3 of the 40 mice.

From the total 5 mice in which the transformation took place, single colonies from blood agar plates were transferred to serum broth, and after incubation at 37° for 24 hours, the fermentation power of the cultures was examined. From 1 mouse (No. 2223) altogether 4 single colonies were established in this way, in order to see whether all the new-formed Type 6 pneumococci behaved alike as to fermentation of the sugars. From each of the 4 other mice only one single colony was examined. Altogether 8 serum broth cultures were examined, 1 drop of blood culture being transferred to each of 12 tubes with inulin, mannite, melibiose and salicin (3 tubes with each sugar). The results are recorded in Table 28.

Table 28 shows that all the new-formed Type 6 pneumococci fermented inulin (100 %) but not mannite (0 %), *i. e.*, their fermentative qualities were exactly like those of the R 1 employed

TABLE 28

Pneumococcus Type 6 (1➤6) Mouse No.	Inulin	Mannite	Melibiose	Salicin
2175	3 + 0 —	0 + 3 —	0 + 3 —	1 + 2 —
2183	3 + 0 —	0 + 3 —	3 + 0 —	3 + 0 —
2233	3 + 0 —	0 + 3 —	1 + 2 —	3 + 0 —
2233	3 + 0 —	0 + 3 —	1 + 2 —	1 + 2 —
2233	3 + 0 —	0 + 3 —	0 + 3 —	1 + 2 —
2233	3 + 0 —	0 + 3 —	0 + 3 —	2 + 1 —
2265	3 + 0 —	0 + 3 —	0 + 3 —	2 + 1 —
2273	3 + 0 —	0 + 3 —	1 + 2 —	0 + 3 —

3 + = 3 tubes which were fermented in 14 days.
2 — = 2 tubes which were not fermented in 14 days.

for the experiment, not like those of the vaccine, Type 6. Melibiose and salicin were fermented by the new-formed Types respectively 6 of the 24 times (25 %) and 13 out of 24 times (54.2 %).

This experiment showed that the new-formed Type 6 fermented the same sugars as the R 1 strain employed, not as the pneumococcus type from which the vaccine was prepared, that is: the transformation cannot be due to surviving pneumococci in the vaccine but must be attributable to the attachment of a capsule made up of capsular substance of the vaccine to the injected R pneumococci.

4. Filtration Experiments.

The explanation of the fact that the capsular substance for one type could be transferred to the body of another type might conceivably be that the pneumococcus culture and the heat-killed vaccine prepared from this culture contained or produced a substance (enzyme? activator?) which brought about the transfer of a capsular substance from one side to another. This substance must be thermolabile, as in no instance could transformation be obtained if the vaccine had been heated to 90° or more; and further, the more and the longer the vaccine had been heated, the less frequently did transformation take place. Attempts were made to isolate this hypothetical substance by filtration of a Type S 2 culture. The filtration was performed through Chamberland L 2 and L 5 filters.

3 experiments were carried out to transform Type 1 to Type 2. An R 1 culture was employed (R 1 obtained from the homologous S 1 after 5 passages in immune serum) together with vaccine S 2 (the vaccine was prepared by autoclaving for 1 hour at 100° in order to destroy the hypothetical thermolabile substance) and filtrate of a Type 2 culture. R 1 and S 2 were respectively sensitive and resistant to M&B with a sensitivity of 6 : 4 and 1 : 1, and the R 1 strain was the same as the one employed for the experiments on transformation of Type 1 to Type 2 in which the transformation succeeded.

The filtrate employed in the first experiment was obtained from a Type S 2 culture by filtration through a Chamberland

L 2 filter. The filtrate was then incubated at 37° for 24 hours, and as it still remained clear, 30 mice were injected subcutaneously with 0.25 cc. of R 1 culture, 0.75 cc. of vaccine S 2 and 0.25 cc. of filtrate. In addition, 10 of the mice were injected with 0.1 cc. of anti-R 1 serum, and 10 mice with 0.1 cc. of anti-S 2 serum. The sterility of the vaccine was tested by subcutaneous injection of the vaccine on 2 mice and by cultivation in the aforementioned media. The sterility of the filtrate was likewise tested by subcutaneous injection on 2 mice and by cultivation in blood broth, serum broth and Truche, as well as on blood agar plate. Both mice which were inoculated with the filtrate survived; on the other hand, living Type 2 pneumococci were demonstrated in vitro in 6 out of 22 tests for sterility.

Even though the filtrate thus was not sterile, no Type 2 pneumococci were demonstrated in the 30 experimental mice, which were autopsied and examined in the usual way.

For the next 2 experiments the filtrate was obtained from a Type S 2 culture by filtration through a Chamberland L 5 filter. The R 1 and S 2 strains were the same as those employed in the preceding experiments. The sterility of the vaccine and the filtrate was examined both in vivo and in vitro, and both were found to be sterile. 25 mice were injected subcutaneously with 0.25 cc. of R 1 culture, 0.75 cc. of vaccine S 2 and 0.25 cc. of filtrate, while 5 mice were injected with 0.5 cc of R 1 culture, 0.75 cc. of vaccine S 2 and 0.1 cc. of filtrate; 15 mice with 0.1 cc. of R 1 culture, 0.75 cc. of vaccine S 2 and 0.25 cc. of filtrate, and 15 mice with 0.1 cc. of R 1 culture, 0.5 cc. of vaccine S 2 and 0.25 cc. of filtrate. In addition, 20 of the mice were also injected with 0.1 cc. of anti-R 1 serum, and 20 mice with 0.1 cc. of anti-S 2 serum. Further, 5 mice were injected subcutaneously with 0.25 cc. of R 1 culture and 0.75 cc. of vaccine S 2, and 5 mice with 0.1 cc. of R 1 culture and 0.75 cc. of vaccine S 2. All the mice were autopsied and examined in the usual way, but in none of the mice did transformation of Type 1 to Type 2 turn out successful.

TABLE 29

R form	S form (vaccine)	No. of mice	No. of mice with transformation of type
Type 1 old	Type 2	127	13
» 1 old	» 8	10	0
» 1 young	» 14	24	0
» 1 young	» 20	24	0
» 1 old	» 22	89	0
» 1 old	» 35	92	1
» 1 sens. young	» 2 res.	100	62
» 1 res. old	» 2 sens.	140	0
» 1 res. young	» 2 sens.	60	18
» 2 sens. young	» 1 res.	20	6
» 2 sens. old	» 1 res.	78	0
» 2 res. young	» 1 sens.	59	6
» 1 sens. old	» 8 res.	40	7
» 1 res. young	» 8 sens.	120	0
» 1 res. old	» 8 sens.	201	0
» 8 sens. young	» 1 res.	80	0
» 8 sens. old	» 1 res.	40	0
» 8 res. young	» 1 sens.	40	0
» 8 res. old	» 1 sens.	80	0
» 1 young	» 6	60	5

res. = resistant.
sens. = sensitive.

5. *Survey of all the Experiments in vivo.*

In Table 29 a survey is given of alle the mouse experiments reported in this chapter. The four columns give the R type employed, the S type, all the mice employed in the transformation experiment, and the number of mice in which the transformation took place. "Old" and "young" affixed to the type of the R form signify that the R form was obtained from the homologous S pneumococcus after more or less than 10 passages in immune serum.

Chapter V.

TRANSFORMATION EXPERIMENTS IN VITRO

As mentioned in Chapter I, 12, these transformation experiments were carried out in two different ways.

One method consisted in mixing the vaccine and serum or blood broth together with anti-R serum in a test-tube, inoculating the tubes with the R culture and incubating them at 37°. Every day or every other day for 15 days a loopful of the mixture was transferred to blood agar plate and to serum broth, and after incubation of these subcultures at 37° for 24 hours they were examined for capsulated pneumococci with the Neufeld reaction by employment of serum of the vaccine's type and serum of the S pneumococcus type from which the employed R pneumococcus originated.

The schema after which this method was employed is illustrated by Table 30.

The other method consisted in mixing the vaccine in test-tubes with serum broth or blood broth and anti-R serum, inoculating the tubes with the R culture and incubating them at 37° for 24-48 hours. After this, 1 loopful of the mixture was transferred to a new series of tubes containing the same amount of vaccine, serum broth or blood broth and anti-R serum; and this procedure was repeated 5-6 times, in one experiment 10 times. At the time of the transfer, 1 loopful of the mixture was spread on blood agar plate and 1 loopful was transferred to serum broth, and after incubation for 24 hours the subcultures were examined for capsulated pneumococci by means of the

TABLE 30

Killed S pn.	Living R pn.	Anti-R 1 serum	Blood broth	No. of tubes	Result
Type 2	Type 1				2 R (7)
0.75 cc.	1 drop	0.3 cc.	1.5 cc.	5	1 R (11)
	dil. 10^{-4}				2 R
Type 2	Type 1				
0.75 cc.	1 drop	0.3 cc.	1.5 cc.	5	1 R (11)
	dil. 10^{-5}				4 R
Type 2	Type 1				2 R (7)
0.75 cc.	1 drop	0.3 cc.	1.5 cc.	5	1 R (9)
	dil. 10^{-6}				2 R
Type 2	Type 1				1 R (7)
0.75 cc.	1 drop	0.3 cc.	1.5 cc.	5	1 R (9)
	dil. 10^{-7}				1 R (11)
					2 R
Type 2	Type 1				
0.75 cc.	1 drop	0.3 cc.	1.5 cc.	5	1 R (9)
	dil. 10^{-8}				4 —
Type 2	Type 1				
0.75 cc.	1 drop	0.3 cc.	1.5 cc.	5	5 —
	dil. 10^{-9}				

R pn. = non-capsular pneumococci.
S pn. = capsular pneumococci.
dil. = dilution.
2 R (7) = from 2 tubes R pneumococci were grown in 7 days.
2 R = from 2 tubes R pneumococci were grown for all 15 days.
4 — = 4 tubes sterile from 1' day.

Neufeld reaction with serum of the vaccine's type and serum of the S pneumococcus type from which the R pneumococcus was obtained.

The schema after which this method was employed is illustrated in Table 31.

The vaccine used for all the experiments in vitro was made of the centrifugate from a 10-12-hour serum broth or ox heart broth culture by heating to 60° for 20 min., and the R pneumo-

TABLE 31

Tube No.	Killed S pn.	Living R pn.	Anti-R 1 serum	Serum broth	Result
1544-1548	Type 2 0.75 cc.	1 drop Type 1 dil. 10^{-4}	0.2 cc.	1.5 cc.	5 R
1544a-1548a	Type 2 0.75 cc.	1 loopful from 1544-1548	0.2 cc.	1.5 cc.	5 R
1544b-1548b	Type 2 0.75 cc.	1 loopful from 1544a-1548a	0.2 cc.	1.5 cc.	5 R
1544c-1548c	Type 2 0.75 cc.	1 loopful from 1544b-1548b	0.2 cc.	1.5 cc.	5 R
1544d-1548d	Type 2 0.75 cc.	1 loopful from 1544c-1548c	0.2 cc.	1.5 cc.	5 R
1544e-1548e	Type 2 0.75 cc.	1 loopful from 1544d-1548d	0.2 cc.	1.5 cc.	5 R

R pn. = non-capsular pneumococci.
S pn. = capsular pneumococci.
dil. = dilution.
5 R = from 5 tubes, R pneumococci were grown.

cocci here employed came from an 8-10-hour serum broth culture. In these experiments in vitro the aim was transformation of Type 1 to Types 2, 8, 22 and 35, and transformation of Type 2 to Type 1.

Experiments on Transformation of Type 1 to Type 2.

Altogether 14 experiments with a total of 324 tubes were carried out after the method shown in Table 30. The first 2 experiments, with 4 tubes, were carried out with R 1 and S 2 pneumococci, both of which strains were sensitive to M&B (sensitivity 6:4). 10 experiments, with 260 tubes, were carried out with the sensitive R 1 (6:4) and the resistant S 2 (1:0); and 2 experiments, with 60 tubes, were carried out with the resistant R 1 (2:2) and the sensitive S 2 (5:4).

The culture medium employed in the first two experiments was 1.0 cc. of ox heart broth, in 1 experiment it was 1.5 cc. of serum broth, and in all the remaining 11 experiments it was 1.5 cc. of blood broth (serum broth with addition of 1 drop of normal

defibrinated rabbit blood). In all the experiments the amount of vaccine added to each tube was 0.75 cc.—with exception of 4 times when only 0.25 cc. of vaccine was added. The amount of R pneumococcus culture added to each tube varied in this way: One drop of undiluted culture was added to 10 tubes, and one drop of dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} was added respectively to 10, 10, 10, 30, 30, 160, 20, 20 and 15 tubes; 0.25 cc. of dilution 10^{-2} was added to 4 tubes; and 1 R pneumococcus colony was transferred from a blood agar plate to each of 5 tubes. The number of R pneumococci in the culture dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} was respectively on an average about 418, 66, 11, 2, 0 and 0.

The first two experiments with 2×2 tubes were carried out with 0.25 cc. of 2 somewhat old anti-R 1 sera with agglutinative titers of 40 and 20, respectively, to which 1 ‰ quinosol had been added; and all 4 tubes were sterile from the first day of the experiment—undoubtedly on account of the addition of quinosol. The remaining 12 experiments were carried out with fresh anti R 1 serum with a titer of 640-1280, without addition of any disinfectant. 0.3 cc., 0.2 cc. and 0.1 cc. of anti-R 1 serum was added respectively to 60, 120 and 20 tubes. Further, 0.1 cc. of the dilutions 1:10, 1:20, 1:40, 1:80 and 1:160 was added respectively to 20, 30, 40, 20 and 10 tubes.

The R 1 strain employed in these experiments was obtained from the homologous S 1 by cultivation in immune serum, as follows: in 1 experiment with 30 tubes after 1 passage, in 3 experiments with 70 tubes after 2 passages, in 1 experiment with 20 tubes after 3 passages, in 1 experiment with 20 tubes after 4 passages, in 4 experiments with 120 tubes after 5 passages, in 2 experiments with 60 tubes after 7 passages, in 1 experiment with 2 tubes after 31 passages, and in 1 experiment with 3 tubes after 32 passages.

In none of the experiments was Type 1 transformed to Type 2. Three times experiments were carried out in vivo on the same day as those in vitro, with employment of the same R strain and the same vaccine, and the transformation of type was successful in 2 of these 3 experiments in vivo.

No less than 37 of the tubes proved sterile from the first

day of observation, namely: 14 out of the 15 tubes inoculated with 1 drop of the R culture in dilution 10^{-9}, 16 of the 20 tubes inoculated with 1 drop of the R culture in dilution 10^{-8}, 5 of the 20 tubes inoculated with 1 drop of R culture in dilution 10^{-7}, and 2 out of 160 tubes inoculated with 1 drop of R culture in dilution 10^{-6}. 8 tubes became sterile 3 days after the inoculation with the R culture, 1 tube 4 days, 37 tubes 5 days, 1 tube 6 days, 55 tubes 7 days, 14 tubes 8 days, 47 tubes 9 days, 7 tubes 10 days, 20 tubes 11 days, 5 tubes 12 days, 6 tubes 13 days and 10 tubes 15 days after inoculation with the R culture, while growth of R pneumococci on blood agar plate and in serum broth was obtained from the remaining tubes even as late as 15 days after the inoculation.

In the experiment with R obtained after one passage of the homologous S 1 in immune serum, growth of capsulated pneumococci of Type 1 was obtained in 5 of the 30 tubes.

In 1 of the experiments with 30 tubes inoculated with 1 drop of R culture in dilution 10^{-6}, after 4 days of incubation 0.1 cc. of the mixture was injected intraperitoneally into 30 mice in order to see whether the mixture now contained any new-formed capsulated pneumococci which would be able to produce a fatal septicemia; but no growth of pneumococci was obtained in the cultures from these mice, 2 of which died of an ordinary infection respectively 12 and 13 days after the intraperitoneal injection, while 28 survived the injection.

In 3 tubes (Nos. 2272, 2275 and 2418), respectively 7, 7 and 3 days after the inoculation with the R 1 culture, a few pneumococci were found to show a slight swelling on examination with Type 2 serum. A few of these colonies were transferred to serum broth and the cultures passed through mice altogether 9 times, and yet no distinct capsular swelling could be demonstrated with Type 2 serum. Furthermore, vaccine and serum were produced in the same manner as mentioned in Chapter I, 4 and 5, and examined for agglutinative titer respectively with serum and vaccine of the S and R forms of Types 1 and 2. With R 1 and R 2 the titer was 640-1280, with S 1 and S 2 only about 20, *i. e.*, the pneumococci grown in the tubes were non-capsulated.

3 experiments on transformation from Type 1 to Type 2 with a total of 90 tubes were carried out in the same manner as shown in Table 31. Each experiment was performed with 30 cc. of vaccine S 2 heated in 6 ampullas of 5 cc. All the vaccine was produced at the same time and kept in incubator at 37° till its use. The R 1 and S 2 strains employed for these experiments were respectively sensitive and resistant to M&B, with sensitivity 6 : 4 and 0 : 0. In the first experiment each tube contained 1.5 cc. of serum broth, 0.75 cc. of vaccine S 2 and 0.2 cc. of anti-R 1 serum. The first 5 tubes were inoculated with 1 drop of the R 1 culture in dilution 1 :80, and after the tubes had been incubated at 37° for 24 hours the next 5 tubes were inoculated herefrom, and so on, for the following 5 days; at the same time, subcultures were made from each tube on blood agar plate and in serum broth.

In the next 2 experiments each tube contained 1.5 cc. of culture medium (serum broth in one experiment, blood broth in the other), 0.75 cc. of vaccine S 2 and 0.2 cc. of anti-R 1 serum.

In both experiments the first 5 tubes were inoculated with 1 drop of R 1 culture in dilution 10^{-4}, and transplants from one series of tubes to the next were made every other day.

The R 1 strain employed in all three experiments was obtained from S 1 after 5 passages in homologous immune serum.

In one experiment, 0.5 cc. from each of the last five tubes after the fifth transplant was injected intraperitoneally into 5 mice; and all the 5 mice survived the injection.

In none of the three experiments did the attempt at transformation of Type 1 to Type 2 turn out successful. In one experiment 2 tubes became sterile on the third day after the second transplant, 1 tube on the fifth day after the fourth transplant, and the last 2 tubes on the sixth day after the fifth transplant. In the other two experiments, growth of R pneumococci was obtained from all 60 tubes.

Experiments on Transformation of Type 2 to Type 1.

Altogether 4 experiments with a total of 120 tubes were carried out with R 2 and vaccine S 1, in the same manner as

shown in Table 30. Two of the experiments were performed with the sensitive R 2 and the resistant S 1 strains with a respective sensitivity for M&B of 5:5 and 2:1, while 2 experiments were made with the resistant R 2 and the sensitive S 1, with a respective sensitivity of 0:0 and 5:5.

Each tube contained 1.5 cc. of blood broth with an addition of 0.75 cc. of vaccine S 1. The amount of anti-R 2 serum varied: 0.2 cc. was added to 60 tubes, 0.1 cc. to 10 tubes, and to the remaining 50 tubes 0.1 cc was added in concentrations of 1:2, 1:4, 1:8, 1:10, 1:16, 1:32, 1:50, 1:100, 1:200 and 1:400, each concentration being added to 5 tubes.

Of the 120 tubes, 70 were inoculated with 1 drop of R 2 culture in dilution 10^{-6}, the remaining 50 tubes with 1 drop of R 2 culture in dilutions 10^{-4}, 10^{-5}, 10^{-7}, 10^{-8} and 10^{-9}, each concentration being added to 10 tubes. 1 drop of R culture in dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} contained on an average respectively about 883, 123, 12, 1, 0 and 0 pneumococci.

The 4 experiments were carried out with R 2 pneumococci obtained from S 2 after respectively 4, 5, 6 and 8 passages in homologous immune serum.

In none of the experiments was transformation of Type 2 to Type 1 successful. No less than 22 of the tubes proved sterile on the first day of examination, namely: all the 20 tubes inoculated with 1 drop of R 2 culture in dilutions 10^{-8}, 10^{-9}, and 2 of the 10 tubes inoculated with R 2 culture in dilution 10^{-7}. In the experiments with R 2 obtained from S 2 after 4 and 8 passages in homologous immune serum, capsulated pneumococci of Type 2 were grown in 2 out of 60 tubes, which were inoculated with 1 drop of R 2 culture in dilutions 10^{-4} and 10^{-7}, respectively. 1 tube became sterile 1 day after inoculation with the R culture, 13 tubes 5 days, 25 tubes 7 days, 9 tubes 9 days, 10 tubes 11 days, 7 tubes 13 days, and 6 tubes 15 days after inoculation, while R pneumococci were grown on blood agar plate and serum broth from the remainder of the tubes even 15 days after their inoculation.

In the experiments with R 2 obtained from S 2 after 5 passages in immune serum, mice were inoculated intraperitoneally with 0.1 cc. of the mixture from 5 of the tubes in order to see

whether capsulated new-formed pneumococci could be demonstrated; but all the mice survived the inoculation.

Experiments on Transformation of Type 1 to Type 8.

One experiment with a total of 14 tubes was carried out in the same manner as outlined in Table 30. The R 1 strain here employed was obtained from the homologous S 1 after 49 passages in immune serum; it was resistant to M&B while the S 8 strain was sensitive, their sensitivity being respectively 1:0 and 6:4. In each tube 2 cc. of serum broth was placed together with 0.3 cc. of anti-R 1 serum; to 7 tubes 1 cc. of vaccine S 8 was added, to the remaining 7 tubes 1 cc. of SSS 8 (solution 1:100). All the tubes were inoculated with 0.1 cc. of R 1 culture, and for the following 8 days subcultures were daily made from all the tubes on blood agar plate and in serum broth. In none of the tubes did transformation of Type 1 to Type 8 turn out successful, the growth from all the tubes showing only R pneumococci. Simultaneously with this experiment, another experiment was performed in vivo with employment of the same R 1 strain and the same vaccine S 8; and this experiment turned out negative too as to transformation of type.

Three experiments were carried out in the same manner as shown in Table 31. Two of these experiments were each carried out with 25 cc. of vaccine heated in 5 ampullas of 5 cc. All the vaccine was produced in one batch and incubated at 37° till its use. For the third experiment, altogether 50 cc. of vaccine, heated in 10 ampullas, was employed; this vaccine was produced in two batches on the first and fifth day of the experiment. The R 1 and S 8 strains were respectively resistant and sensitive to M&B with sensitivity 1:0 and 6:4. In the first experiment the tubes contained 2 cc. of serum broth, 1 cc. of vaccine S 8 and 0.3 cc. of anti-R 1 serum. The first 5 tubes were inoculated with 1 drop of R 1 culture in dilution 1:60, and after incubation of the tubes at 37° for 24 hours, transplants were made herefrom to the next 5 tubes, and so on, for the following 4 days.

The R 1 employed in this experiment was obtained from S 1 after 57 passages in homologous immune serum.

The second experiment was carried out in the same way as the preceding, only that anti-R 1 serum was replaced by 0.3 cc. of swine serum, which with R 1, R 2 and R 8 vaccine had an agglutinative titer of 40, 40 and 20 respectively; and the R 1 strain here employed was obtained from S 1 after 62 passages in homologous immune serum. Transformation of Type 1 to Type 8 did not take place in any of the 50 tubes, and growth of R pneumococci was obtained from all the tubes. 4 tubes became sterile on the third day after the inoculation, 1 tube on the fourth day and 2 tubes on the fifth day. From 3 of the tubes, after the fourth transplant, 0.5 cc. of the mixture was injected intraperitoneally into mice; one mouse died on the first day, from an ordinary infection, while the remaining two survived the inoculation.

In the third experiment each tube contained 2 cc. of serum broth, 1 cc. of vaccine S 8 and 0.5 cc. of anti-R 1 serum. The first 5 tubes were inoculated with 1 drop of R 1 culture in dilution 1:60. This experiment was carried out like the others, except that here the daily transplants had continued for 10 days. The R 1 strain employed was obtained from S 1 after 62 passages in homologous immune serum. In none of the 50 tubes did transformation of Type 1 to Type 8 turn out successful, growth of R pneumococci being obtained from all the tubes. 1 tube became sterile on the ninth, 1 tube on the tenth day. After the 9' transplant 0.5 cc. from three of the tubes was injected intraperitoneally into mice. One mouse died on the first day, one mouse on the second day, and the third mouse survived the inoculation. No capsulated pneumococci were demonstrated in the two dead mice. Cultures from one of the dead mice showed growth of R colonies, which were transferred to serum broth and again injected into a mouse, but also this mouse failed to show S pneumococci.

Experiments on Transformation of Type 1 to Type 22.

One experiment with 10 tubes was carried out in the same way as in Table 30. The employed R 1 strain was obtained from S 1 after 50 passages in immune serum. Each tube contained 2 cc. of serum broth, 1 cc. of vaccine S 22 and 0.3 cc. of anti-R 1 serum. All the tubes were inoculated with 1 drop of R 1 culture in dilution 1 : 60, and on the following 8 days transplants were made daily from the tubes on blood agar plate and to serum broth, but none of the 10 tubes showed any transformation of Type 1 to Type 22, only growth of R pneumococci being obtained from all the tubes. Of these tubes 3 became sterile on the fifth day, 2 on the sixth day and 3 on the eighth day. Simultaneously with this experiment another experiment was carried out in vivo with employment of the same R 1 strain and the same vaccine S 22, but also this experiment failed to show any transformation of Type 1 to Type 22.

Then 2 experiments were carried out in the same manner as shown in Table 31, with employment of 25 and 20 cc. of vaccine S 22, respectively, heated in 5 and 4 ampullas. All the vaccine used in each experiment was produced in one batch and incubated till its use. The R 1 strain here employed was obtained from S 1 after 44 and 46 passages in homologous immune serum. Each tube contained 2 cc. of serum broth and 1 cc. of vaccine S 22. In the first experiment 0.3 cc. of anti-R 1 serum was added to each tube, and in the second experiment 0.3 cc. of swine serum. In each experiment the first 5 tubes were inoculated with 1 drop of R 1 culture in dilution 1 : 60, and after incubation of the tubes at 37° for 24 hours, transplants were made from these tubes to the next 5 tubes, and so on—in the first experiment for the following 5 days, in the second experiment for 4 days. Simultaneously with the transplants, subcultures were made on blood agar plate and in serum broth. In none of the 45 tubes was transformation of Type 1 to Type 22 successful, only growth of R pneumococci being obtained from all the tubes. One of the tubes became sterile on the fourth day. After the first transplant in the first experiment and after the third transplant in the second ex-

periment, 0.5 cc. from all the tubes was injected intraperitoneally on mice, 3 of which died on the first day, and one on the second day, while the remaining 6 mice survived the injection. All the mice were autopsied and examined in the usual manner, but no capsulated pneumococci could be demonstrated.

Experiments on Transformation of Type 1 to Type 35.

One experiment with altogether 14 tubes was carried out in the same way as given in Table 30. The R 1 strain was obtained from S 1 after 45 passages in homologous immune serum. Each test-tube contained 2 cc. of serum broth and 0.3 cc. of anti-R 1 serum. 1 cc. of vaccine S 35 was added to 7 tubes, and 1 cc. of SSS 35 (solution 1 : 100) to 7 tubes. All the tubes were inoculated with 1 drop of R 1 culture, and on each of the following 8 days transplants were made on blood agar plate and in serum broth. In none of the tubes did the transformation of Type 1 to Type 35 turn out successful, only growth of R pneumococci being obtained from all the tubes. 2 of the tubes became sterile on the fourth day, 2 on the fifth day and 3 on the sixth day. Simultaneously with this experiment, another experiment was carried out in vivo with employment of the same R 1 strain and the same vaccine S 35; this experiment too turned out negative as to transformation of type.

Then 3 experiments were carried out in the same way as shown in Table 31 with employment of 25, 25 and 20 cc. of vaccine S 35 respectively. All the vaccine used in each experiment was produced in one batch and kept in incubator till its use. The R 1 employed here was obtained from S 1 after 46, 46 and 53 passages in immune serum. Each tube contained 2 cc. of serum broth and 1 cc. of vaccine S 35. In 2 experiments 0.3 cc. of anti-R 1 serum was added to each tube; in the third experiment, 0.3 cc. of swine serum. In each experiment the first 5 tubes were inoculated with 1 drop of R 1 culture in dilution 1 : 60 and after incubation of the tubes for 24 hours, transplants were made from them to the next 5 tubes, and so on—in 2 experiments for the following 5 days, in the third experiment for 4 days. Simultane-

ously with the transplants, subcultures were made on blood agar plate and in serum broth. In none of the 70 tubes did the transformation of Type 1 to Type 35 turn out successful, only growth of R pneumococci being obtained from all the tubes. One of the tubes became sterile on the second day, 2 tubes on the third day, 2 on the fourth day and 1 on the fifth day.

In the experiment with 4×5 tubes, after the third transplant, mice were inoculated with 1 cc. from the tubes. Of these mice, 1 died on the first day, 1 on the second and 1 on the fourth day, but cultures from the mice showed growth only of R pneumococci. Colonies of R pneumococci from one of the dead mice were transplanted in serum broth, and 0.5 cc. of this culture was injected intraperitoneally into a mouse, but also this animal died of R pneumococcus sepsis, and no growth of capsulated pneumococci was obtained in cultures from this animal.

Chapter VI.

SEROLOGICAL STUDIES ON THE NEW-FORMED TYPES AND THEIR CONSTANCY

The following experiments were aimed to show whether the new-formed S form serologically corresponded to the S pneumococci employed for the vaccines in the transformation experiments. Hence cross-absorption was performed between Types S 2 and the new-formed Type S 2 (from Type 1), between S 8

TABLE 32

Vaccine	Pneumococcus sera											
	S 1				S 2				S 2 (1➝2)			
	non-abs.	absorbed with			non-abs	absorbed with			non. abs.	absorbed with		
		S 1	S 2	S 2 (1➝2)		S 1	S 2	S 2 (1➝2)		S 1	S 2	S 2 (1➝2)
S 1	++	—	++	++	—	—	—	—	—	—	—	—
S 2	—	—	—	—	++	++	—	—	++	++	—	—
S 2 (1—➝2)	—	—	—	—	++	++	—	—	++	++	—	—

S 2 (1—➝2) = new-formed capsular Type 2 pneumococcus formed from Type 1.
non-abs. = non-absorbed pneumococcus serum.
++ = strong, macroscopic agglutination and distinct capsular swelling.
-- = no agglutination or capsular swelling.

TABLE 33

Vaccine	Pneumococcus sera											
	S 1				S 8				S 8 (1➤8)			
	non-abs.	absorbed with			non-abs.	absorbed with			non-abs.	absorbed with		
		S 1	S 8	S 8 (1➤8)		S 1	S 8	S 8 (1➤8)		S 1	S 8	S 8 (1➤8)
S 1	++	—	++	++	—	—	—		—	—	—	—
S 8	—	—	—	—	++	++	—	—	++	++	—	—
S 8 (1 ➤8)	—	—	—	—	++	++	—	—	++	++	—	—

TABLE 34

Vaccine	Pneumococcus sera											
	S 1				S 35				S 35 (1➤35)			
	non-abs.	absorbed with			non-abs	absorbed with			non-abs.	absorbed with		
		S 1	S 35	S 35 (1➤35)		S 1	S 35	S 35 (1➤35)		S 1	S 35	S 35 (1➤35)
S 1	++	—	++	++	—	—	—	—	—	—	-	—
S 35	—	—	—	—	++	++	—	—	++	++	—	—
S 35 (1➤35)	—	—	—	—	++	++	—	—	++	++		—

S 8 (1 ➤8) = new- formed, capsular Type 8 pneumococcus formed from Type 1.
S 35 (1➤35) = new-formed, capsular Type 35 pneumococcus formed from Type 1.
non-abs. = non-absorbed pneumococcus serum.
++ = strong, macroscopic agglutination and distinct capsular swelling.
— = no agglutination or capsular swelling.

and the new-formed Type 8 (from Type 1) and between S 35 and the new-formed Type 35 (from Type 1). For control, cross-absorption was carried out simultaneously with the heterologous Type 1. The results are recorded in Tables 32, 33 and 34.

All the absorptions were performed twice, and the agglu-

tinative and capsular titers with the heterologous and homologous vaccines were determined both before and after the absorption. The titers are not given in the tables, in which it is merely recorded whether the non-absorbed sera or the absorbed sera gave a strong agglutination and distinct capsular swelling with the vaccines (++), or gave no agglutination or capsular swelling (—).

From the experiments with cross-absorption it will be noticed that the new-formed pneumococci serologically correspond perfectly to the pneumococci employed for the vaccines in the respective experiments (S 2 = S 2 (1→2), S 8 = S 8 (1→8) and S 35 = S 35 (1→35)).

Like the original Type 35, the new-formed Type 35 was agglutinated by S kirkee serum (O serum), because—as reported previously by *Kauffmann* and *Langvad-Nielsen* (48)—S kirkee bacteria and pneumococcus Type 35 have an antigen in common. In the pneumococci this antigen is located in the capsule, while in S kirkee bacteria it is located in the body.

In the experiments here performed the capsular titer was nearly always somewhat higher than the agglutinative titer. After absorption of the serum with the heterologous vaccine, both the agglutinative and the capsular titers with the homologous vaccine had fallen off 1 or 2 tubes, but both the agglutination and the capsular swelling were always distinct anyhow. The fall in titer was attributable to the dilution associated with the repeated mixing of serum and vaccine.

The new-formed pneumococcus—made up of the body of one type and the capsule of another type will in future behave as the type from which its capsular substance originated, as it has been found to preserve its characteristics of type both on storage and after animal passages. After storage for 2 years in Truche and after numerous mouse passages, the capsular swelling with homologous serum remained just as distinct as before.

Chapter VII.

DISCUSSION

The term "pneumococcus types" signifies serological units with different capsular substances, which under natural conditions are not interchangeable, so that practically they may be considered constant characteristics.

No wonder, therefore, that *Griffith* (38) in 1928 caused a good deal of sensation when he reported that he had succeeded in transforming one pneumococcus type to another type by simultaneous subcutaneous inoculation of mice with small amounts of living non-capsulated pneumococci (R form) of one type, together with large amounts of heat-killed capsulated pneumococci (S form) of another type.

After injection of R 1 pneumococci and vaccine S 2, *Griffith* obtained living pneumococci of Type 2 from the mice treated in this way.

In such experiments the non-capsulated Type 1 pneumococcus must have acquired the capsule from the heat-killed but capsulated Type 2 pneumococcus. Schematically this transformation may be put down as the following formula:

$$\text{R 1} + \text{vaccine 2} = \text{S 2}.$$

Naturally the main objection to such a transformation of type will be that the vaccine was not sterile (the pneumococci of the vaccine were killed by heating to 60° for 20 min.) and that the growth of Type 2 pneumococci in the cultures from the mice originated from surviving pneumococci in the vaccine.

Numerous investigations were carried out to prove the sterility of the vaccine. *Griffith* himself performed a great number of tests for sterility of the vaccine in vivo and in vitro by subcutaneous and intraperitoneal injection of the vaccine on mice and by cultivation in various media, but living pneumococci were never demonstrated in the vaccine. Numerous other investigators—*e.g. Neufeld & Levinthal* (68), *Reimann* (77), *Dawson* (21)—who had confirmed the findings reported by *Griffith* were likewise unable to demonstrate living pneumococci in the heated vaccine by various tests in vivo and in vitro.

On the other hand, *Haendel & Lange* (40) stated that some pneumococci were able to survive heating to 60° for 30 min. and a few times even to 70° for 30 min., as they had obtained growth of pneumococci from the heated vaccine both on cultivation in various media and on injection into mice. In particular, however, *Dunlop* (29) claimed that the vaccine employed for the transformation experiments was not sterile. He examined the vaccine by transplants on 5 % blood agar plate, in 5 % blood broth and in 10 % serum broth, and by leaving the cultures in incubator at 37° for a long period. In this way *Dunlop* thought he had obtained growth of pneumococci in several tests. The description of the experiments carried out by *Dunlop* is defective, however, and the cultivated pneumococci were not examined thoroughly; for instance, he failed to mention to what temperature and for how long he had heated the vaccine; nor did he state how much vaccine was added to each tube of serum broth or blood broth or spread on the blood agar plates. Furthermore, he was unable in subcultures on blood agar plate to obtain growth of pneumococci from the tubes inoculated with vaccine and in which he stated that growth had taken place; nor did he mention the characteristic appearance of his Type 3 colonies on blood agar plate but stated merely that morphologically they resembled pneumococci. Finally, he failed to examine the cultivated pneumococci for capsular swelling with type-specific serum, and most of his pneumococci were either non-sensitive to optochin or not bile-soluble, and none of them were virulent for mice.

In order to prove that transformation of type has taken place, it is necessary with absolute certainty to show that the vaccine

employed was sterile or, preferably, prove that the body of the new-formed pneumococcus was the injected R strain.

Prior to my studies on the sterility of the vaccine, I investigated to which temperature the pneumococci had to be heated in order to be killed. The cultures were heated in sealed ampullas of glass to temperatures from 47° to 60°, the ampullas being entirely immersed in a water-bath which was automatically kept at the desired temperature, and after this heating transplants were made from the ampullas in a number of different culture media, and the vaccine was also injected subcutaneously into mice. These experiments showed that the pneumococci were killed on heating to 60° for 20 min., and also that pneumococci grown in a serum protein-containing medium (serum broth) had to be heated to a higher temperature in order to be killed than did pneumococci grown in a non-serum-containing medium (ox heart broth); besides, the denser the suspension of pneumococci, the higher the temperature required to kill all the pneumococci.

In my experiments the vaccine was prepared from the centrifugate of pneumococcus cultures heated in sealed ampullas in water-bath to 60° for 20 min. The sterility of the vaccine was tested, partly in vitro (2917 times) by cultivation in a number of different media under anaerobic as well as aerobic conditions and in CO_2 atmosphere, partly in vivo (533 times) by subcutaneous injection on white mice. The mice were injected partly with the heated vaccine alone (223 times), partly with vaccine together with living bacteria (colon bacilli, streptococci, staphylococci) or with a solution of the specific polysaccharid of the pneumococci (SSS), or the vaccine was injected into animals treated preliminarily with mucin or alcohol (310 times). If living pneumococci were present in the vaccine, their virulence might conceivably have been lowered to such an extent that they were unable to kill the mice and then were killed themselves in the mice. But when the vaccine was injected with living R pneumococci (in the transformation experiments) or other bacteria or foreign substances (in the tests for sterility in vivo), the resistance of the mice would perhaps be lowered to such an extent

that the injured and attenuated S pneumococci in the vaccine were now able to multiply in the mice and kill them.

All these experiments turned out negative, however, with exception of the two aforementioned cases which do not allow of any definite conclusion.

But even though it is warrantable, apart from very rare exceptions, to look upon the vaccines employed as sterile, the evidence of the transformation of type would be more conclusive by demonstrating that the body of the new-formed pneumococcus was the same as the R strain employed in the experiment. For this purpose my experiments were carried out with marked pneumococcus strains, *i. e.*, strains with established constant properties associated with the body of the pneumococcus: chemoresistance and fermentative qualities—a measure which, as far as I have been able to find out, has not been adopted by any previous investigator. These two properties, chemoresistance and fermentation power, were not influenced by storage, mouse passage or culture passage in serum broth. The chemoresistance of the pneumococci was estimated for sulfapyridine (M&B) by employment of the technique given by *Schmith* (79) and the fermentation power was determined for 12 "sugars" added to Hiss' medium.

After subcutaneous inoculation of mice with a chemoresistant R form of one type (*e. g.*, Type 1) together with a sensitive S vaccine of another type (*e. g.*, Type 2), I was able in cultures from the mice to obtain growth of Type 2 pneumococci with the same resistance as the injected R pneumococci. This experiment indicated that a transformation of Type 1 to Type 2 had taken place, but the opposite experiment with sensitive R 1 and resistant S 2 had to be carried out before such a transformation of type could be stated with certainty to have taken place; for it was conceivable that the S pneumococci of the vaccine under the heating might have become resistant to M&B. If so, the pneumococci cultivated from the mice might just as well originate from surviving pneumococci in the vaccine. But as the experiment with the sensitive R form of Type 1 and the resistant

S vaccine of Type 2 yielded growth of sensitive Type 2 pneumococci in cultures from the mice, it could be said with certainty that a transformation of type had taken place or, rather, that the capsular substance of the vaccine had been attached to the R pneumococci. For it is hardly conceivable that the pneumococci under heating in one case would change in sensitivity from being sensitive to becoming resistant and in another case from being resistant to becoming sensitive when the experimental conditions otherwise were the same.

Besides the transformation of Type 1 to Type 2, also transformation of Type 2 to Type 1 was successful both with R 2 sensitive and vaccine S 1 resistant and also with R 2 resistant and vaccine S 1 sensitive, and transformation of Type 1 to Type 8 with R 1 sensitive and vaccine S 8 resistant; and all the new-formed pneumococci grown in cultures from the mice possessed the same resistance as the injected R pneumococci. On the other hand, the attempts at transformation failed in the experiments with R 1 resistant and vaccine S 8 sensitive, with R 8 sensitive and vaccine S 1 resistant, and with R 8 resistant and vaccine S 1 sensitive.

Just as the resistance of the pneumococci to M&B is a property of the body of the pneumococcus, also the fermentation power is associated with the body of the pneumococcus, as the experiments showed that the R pneumococci fermented the sugars essentially like the homologous S pneumococci. On the new-formation of pneumococci by means of the R and S forms of two pneumococcus types with different fermentative qualities, it should be possible from the fermentation power of the new-formed types to decide whether a transformation of type actually had taken place. If the new-formed pneumococci fermented like the injected R pneumococci, a change of capsule had taken place; if, on the other hand, they fermented like the S pneumococci of the vaccine, surviving pneumococci would have been present in the vaccine.

The fermentation experiments here presented showed that R 1 pneumococci always fermented inulin and never mannite, whereas Type 2 never fermented inulin and nearly always mannite. The new-formed Type 2 pneumococci, resulting from R 1

and vaccine S 2, fermented always inulin and never mannite. Similarly, Type 6 never fermented inulin, but the new-formed Type 6, resulting from R 1 and vaccine S 6 fermented inulin in every test, *i. e.*, the new-formed pneumococci fermented exactly like the injected R 1 pneumococci. In the experiments on transformation of Type 1 to Types 8 and 35, the result was less clear-cut because the difference in the fermentation power of Type 1 and the two other types was very slight; still, the new-formed Types 8 and 35 did not ferment mannite in any test—just like R 1 pneumococci—whereas the original Types 8 and 35 fermented mannite respectively in 50 % and 25 % of the tests. Further, the experiments showed that the R form of the new-formed Type 2 pneumococcus fermented exactly like R 1 pneumococci, not like R 2.

The experiments concerning the resistance of pneumococci to M&B and their fermentation power showed that the new-formed type received its type-specific properties from the capsular substance of the vaccine employed, whereas the species-specific properties originated from the R strains. The experiments further showed that the transformation of type took place gradually, as on examination for capsular swelling with the homologous immune serum, some of the new-formed pneumococci showed a distinct capsular swelling, while others presented merely a slight thickening of the capsule, and all sorts of transitions between these two forms were seen. Moreover, the capsular substance appeared not always to be distributed equally, as some of the pneumococci had "bumps" in their capsular swelling. The appearance of the colonies of the new-formed pneumococci on blood agar plate was likewise subject to some variation, as the plates showed both large colonies which completely resembled the colonies of the S pneumococci employed for the vaccine and small colonies that were difficult to distinguish from the R colonies. Finally, the new-formed types were virulent to mice and presented all the other properties of the pneumococcus.

Experiments in vivo were carried out altogether on 1484 mice, but the attempt at transformation did not turn out successful in all the mice. When the experiments were carried out with R forms obtained from S pneumococci after a few passages in

homologous immune serum the transformation proceeded more readily; and in some of these experiments the transformation of type took place in 100 % of the mice, while on an average the outcome was successful in 32.5 %. When very young R pneumococci were employed, some R pneumococci would revert to the homologous type and, in the same mouse, others would be transformed to the heterologous type, while finally some would remain as the R form. If the R pneumococci were stable, *i. e.*, derived from S pneumococci after many passages in immune serum, the transformation experiment would as a rule turn out negative —in my experiments, under such conditions transformation of type took place on an average in 4.4 % of the mice. When the transformation of Type 1 to Types 14 and 20 was unsuccessful, although the R strain employed in these experiments was obtained from S 1 after only 5 passages in homologous immune serum, this result was undoubtedly due to the circumstance that the proportion between the injected amounts of R 1 culture and vaccines S 14 and 20 was not optimal, as most of the mice died of R pneumococcus sepsis; besides, these experiments were carried out with relatively few mice, 48 altogether. It is difficult to say why the transformation of Type 1 to Type 8 with R 1 resistant and vaccine S 8 sensitive turned out a failure, as the R 1 strain was unstable—the very same strain as had been employed in the experiments on transformation of Type 1 to Type 2, which turned out successful. But it may be that in none of the experiments was the proportion between the injected amounts of R 1 and S 8 optimal. When R 8 could not be transformed to Type 1, it was undoubtedly because S 8 formed all too unstable R forms on cultivation in homologous immune serum.

Several attempts were made to isolate a hypothetical thermolabile substance which might transfer the capsular substance from one type to the body of another type. Thus 100 mice were injected subcutaneously with R culture, S vaccine (autoclaved for 1 hr. at 100°) and filtrate from a living S culture, but none of the mice yielded any capsulated pneumococci of the same type as the vaccine. This negative outcome of the few orientating experiments allows of no definite conclusion.

The transformation of type in vitro, which turned out suc-

cessful for *Dawson & Sia* (25-26), *Dawson & Warbasse* (27), *Baurhenn* (8) and others, was unsuccessful in my experiments. As my experiments were carried out in the same way as recorded by *Dawson & Sia*, I am not able to offer any adequate explanation of these negative results. The R strain employed here was young, the number of R pneumococci added to the tubes was small, and the anti-R serum was added in varying amounts of differing concentration.

For additional corroboration of the transformations of type obtained in these experiments, cross-absorption was carried out between 4 different R forms, between the new-formed S forms and the S pneumococci employed for the vaccine, and between the R forms of the new-formed S pneumococci and the R forms employed in the experiment. In this way it was found that the R forms of all pneumococcus types were serologically identical, that the new-formed S pneumococci serologically corresponded to the S types employed for the vaccine, and that the R forms of the new-formed S pneumococci were identical with the R forms employed in the experiment. The new-formed types remained constant, as their capsules kept unchanged even on preservation for a considerable length of time (examined for up to 2 years) and after numerous mouse passages.

The transformation of type here described is actually a new-formation, as the new type is built up of the corpus of one type and the capsule of another type, and it possesses the corporal qualities of the one type and the capsular properties of another type.

The corpus (R form) of the various pneumococcus types may vary as to chemoresistance and fermentation power, but all the corpora are serologically identical. An R pneumococcus which has the capsule of a heterologous type attached to it, will in future appear as the type from which the capsular substance is derived, the new type preserving its properties on storage and continued cultivation in vivo and in vitro.

Chapter VIII.

SUMMARY

Part I. Historical Survey.

A brief survey is given of the discovery, typing, type diagnosis and fermentative diagnosis of the pneumococci. Further, mention is made of the variation of the pneumococci due to unfavorable conditions in the surroundings. The pneumococci may lose their virulence and capacity for capsule formation, so that their type-specificity is lost but the species-specificity is preserved. A virulent, capsulated type-specific pneumococcus is designated as the S form of the organism, and the avirulent noncapsulated and non-type-specific pneumococcus is designated as the R form.

The variation of the pneumococci has been investigated especially by cultivation in homologous immune serum (*Stryker* (84), *Griffith* (37), *Reimann* (75-76), *Dawson* (19-20) and others). Concentrated serum is not required for dissociation of the pneumococci, a serum dilution of 1 : 256 being sufficient to produce a partial change of S pneumococci to R pneumococci, but the optimal serum concentration for this purpose is 10-25 %. The reversion from R to S takes place in vitro on cultivation in 10 % anti-R serum, in vivo on subcutaneous inoculation of white mice with large amounts of living R culture or small amounts of R culture together with large amounts of heat-killed homologous S culture.

Further, mention is made of the transformation of pneumococci from one Type to another, which was first reported by *Griffith* (38), and later by *Reimann* (77, *Dawson* (21), *Baurhenn*

(8), *Alloway* (1-2) and others. *Griffith* was the first to be successful in the transformation of type in vivo, when he inoculated mice subcutaneously with R pneumococci of one type together with heat-killed S pneumococci of another type. *Dawson & Sia* (25, 26, 80), were successful in transformation of type in vitro by transplanting minimal amounts of an R culture to test-tubes containing blood broth, heat-killed S pneumococci and anti-R serum. *Alloway* (1-2), obtained transformation of type in vitro when the S vaccine was replaced by filtered extract of the pneumococci.

Finally, mention is made of investigations reported by various authors on the influence of the temperature, and most of these authors hold that the pneumococci are killed by heating to 60° for 20 min. (*Lange* (54), *Paul* (74), *Avery* (6), *Stryker* (84), *Baurhenn* (8), *Griffith* (38), *Reimann* (77), *Dawson* (20-21) and many others), whereas a few (*Handel & Lange* (40), *Dunlop* (29)) think that heating to 60° for a longer period or to a temperature higher than 60° is required in order to ensure that all the pneumococci are killed.

Part II. Writer's Studies.

Chapter I deals with the entirely technical aspects of the experiments: the culture media employed in the tests for sterility of the vaccine, the cultures employed in the various experiments, determination of the virulence of the capsulated pneumococci for white mice, production of S and R vaccines, the specific soluble substance of the pneumococci, immune serum and R pneumococci; further, the performance of the tests for sterility in vivo and in vitro, experiments on transformation of type in vivo carried out in the same manner as reported by *Griffith* (38), and the performance of the M&B experiments, the fermentation experiments and the filtration experiments; finally, the experiments on transformation of type in vivo carried out with the technique given by *Dawson & Sia* (25, 26), and the performance of the absorption experiments.

Chapter II deals with the studies on R pneumococci. On cultivation in homologous immune serum, all S pneumococci form more or less stable R pneumococci, *i. e.*, R pneumococci which

do not revert to the homologous pneumococci on cultivation in serum broth or on intraperitoneal injection into mice. Thus R 8, R 22 and R 35, obtained from the homologous S pneumococci respectively, after 83, 20 and 20 passages, were not stable, whereas R 1 and R 2 were stable already after 5 passages of S 1 and S 2 in immune serum. The R pneumococci behaved towards M&B in the same manner as the homologous S pneumococci; if the latter were resistant, the R forms would be resistant too, and if they were sensitive, the R forms would be sensitive too. All the R pneumococci examined were serologically identical, and the R forms of the new-formed S pneumococci were identical with the R forms employed in the experiment concerned. This identity was proved by cross-absorption with the various R pneumococci. It is further proved that anti-R serum is emptied of agglutinins by absorption both with the homologous R and S pneumococci and with the heterologous R pneumococci, whereas the agglutinins of an anti-S serum are removed only by absorption with homologous S pneumococci, not with the R pneumococci, the latter being able to remove only the species-specific agglutinins not the type-specific.

Chapter III gives an account of studies on the sterility of vaccine. Pneumococci grown in serum protein-containing media had to be heated to a higher temperature if all the organisms were to be killed than was required for pneumococci grown in media that did not contain serum protein. The denser the suspension of pneumococci, the higher the temperature that was required in order to kill them all. In no instance, however, was growth of pneumococci obtained in subcultures after heating of the culture to 60° for 20 min. The vaccine employed for the transformation experiments was produced with heating to 60° for 20 min. Altogether 2917 sterility tests in vitro and 533 sterility tests in vivo were carried out with the vaccine. The tests in vitro were performed by addition of varying amounts of the vaccine to various fluid and solid culture media, which then were incubated under anaerobic as well as aerobic conditions or in CO_2 atmosphere. The tests in vivo consisted in subcutaneous inoculation of white mice either with the vaccine alone or

together with other bacteria (colon bacilli, streptococci, staphylococci, killed R pneumococci) or together with the specific soluble substance (SSS) of the pneumococci; and in some experiments the mice were treated with mucin or alcohol.

Chapter IV gives an account of all the experiments on transformation of type in vivo, which were all carried out by subcutaneous injection of small amounts of R culture of one type together with large amounts of heat-killed S pneumococci (vaccine) of another type. With the technique given by *Griffith* the transformation of Type 1 to Types 2 and 35 was successful, whereas the attempts at transformation of Type 1 to Types 8, 14, 20 and 22 were unsuccessful. The new-formed Type 35 possessed the same virulence as the Type 35 of the vaccine, not like that of Type 1 from which it was formed.

In order to prove that the new-formed pneumococci consisted of the same body as the injected R pneumococci, marked strains were employed, *i. e.*, strains with established constant properties connected with the body—as chemoresistance and fermentation power. These two properties were not influenced by storage, mouse passages or culture passages in serum broth. After inoculation of a mouse with a chemoresistant R form of one type together with vaccine of the sensitive S form of another type, growth was obtained in cultures from the mouse of chemoresistant S pneumococci of the vaccine type. In order to exclude the possibility that the sensitive S pneumococci became resistant during the process of heating, the experiment was reverted: the sensitive R form was injected together with the chemoresistant S form, and now growth was obtained from the mice of sensitive S pneumococci of the vaccine type. In this way the following experiments turned out successful: R 1 sensitive + vaccine S 2 resistant → Type 2 sensitive; R 1 resistant + vaccine S 2 sensitive → Type 2 resistant; R 2 sensitive + vaccine S 1 resistant → Type 1 sensitive; R 2 resistant + vaccine S 1 sensitive → Type 1 resistant; and R 1 sensitive + vaccine S 8 resistant → Type 8 sensitive.

After inoculation of mice with an R form of one type together with the vaccine of an S form of another type, the fermentation

power of the R form differing from that of the S form, growth was obtained from the animals of capsulated pneumococci of the same type as the S form of the vaccine but with the fermentative qualities of the R form. Thus the new-formed types, 2, 6, 8 and 35, possessed the same fermentation power as that of the injected R 1 pneumococci, different from that of the vaccine type. The R form of a new-formed Type 2 showed the same fermentation power as did the R 1 employed for the experiment, not like the R form of the vaccine type (R 2).

The experiments with pneumococci differing in chemoresistance and fermentation power showed that the new-formed type consisted of the corpus from one type and the capsule from another type, and it possessed the corporal properties of one type and the capsular characteristics of another type.

On blood agar plate, in most instances, the colonies of the new-formed types looked like colonies of the vaccine type, but some of the colonies were small and indistinguishable from R colonies. In tests with immune serum as a rule the capsular swelling was distinct; still, some of the new-formed pneumococci showed only a slight capsular swelling which did not improve on repeated mouse passages. In the same mouse some pneumococci of the new-formed type showed only a slight thickening of the capsule, in others it was only faintly developed, while some presented a fully developed capsule. Some pneumococci presented an uneven capsular swelling, the capsule having some "bumps". In some of the inoculated mice some of the R pneumococci had acquired the heterologous capsule, others the homologous capsule, while some remained R pneumococci.

The experiments showed that on employment of young R pneumococci—*i. e.*, the R form obtained from the homologous S form after a few passages in immune serum—the transformation of one type to another took place more readily (on an average in 32.5 % of the mice) than on employment of old R pneumococci, *i. e.*, the R form obtained from the homologous S form after many passages in immune serum (transformation of type on an average in 4.4 % of the mice).

The fermentation power of the pneumococci was examined in tests with arabinose, xylose, dulcite, inosite, glucose, galactose,

lactose, saccharose, inulin, mannite, melibiose and salicin. The first four "sugars" were never fermented, the next four always, except by a certain Type 11, and the last four were fermented differently by the various pneumococci. The experiment showed that serologically different types did not differ distinctly as to fermentation power, that serological identical strains did not always possess the same fermentation power, and that the R pneumococci essentially fermented like the homologous S pneumococci.

Finally, in this chapter, mention is made of a few experiments on preparation of the hypothetical thermolabile substance which is able to attach the capsular substance of one type to the corpus of another type. 100 mice were injected subcutaneously with R 1 culture, vaccine S 2 (prepared by autoclaving for 1 hr. at 100°) and filtrate of a Type 2 culture, but in none of the mice did transformation of Type 1 to Type 2 turn out successful.

Chapter V deals with the experiments on transformation of type in vitro. Altogether 787 test-tube experiments were carried out in the same way as given by *Dawson & Sia* (25, 26): the tubes containing the culture medium, vaccine and anti-R serum were inoculated with the R culture and incubated at 37°. For the following 15 days subcultures were made daily from the tubes on blood agar plate and in serum broth for examination for capsular pneumococci; or, after incubation for 24 hours, transplants were made to another series of tubes containing the same amounts of culture medium, vaccine and anti-R serum, followed by subcultivation on blood agar plate and serum broth, and so on for the following 4-10 days. As a rule the culture medium consisted in blood broth, but serum broth was used in a few experiments. The vaccine was the centrifugate from ox heart broth or serum broth cultures heated to 60° for 20 min. The anti-R serum, with a titer of 640-1280, was added to the tubes in varying amounts (0.1-0.3 cc.) of varying concentration, from undiluted serum to dilution 1 : 400. Also the amount of R pneumococci added to the tubes varied, 1 drop being added to each tube, from whole culture to a dilution of 10^{-9}. No transformation of type was ob-

tained in any of my experiments (from Type 1 to Types 2, 8, 22 and 35, and from Type 2 to Type 1).

Chapter VI deals with serological studies on the new-formed types and their constancy. By cross-absorption it is shown that the new-formed Type 2 obtained from Type 1 is serologically identical with the ordinary Type 2. Likewise the new-formed Type 8 is serologically identical with the ordinary Type 8, and the new-formed Type 35 with the ordinary Type 35. On storage and through numerous subcultures and mouse passages the new-formed pneumococci preserved their acquired properties both of capsule and of corpus.

RESUMÉ

I. Afsnit. Historisk Oversigt.

Der gives en ganske kort Oversigt over Pneumococcernes Opdagelse, Typeinddeling, Typediagnose og Forgæringsdiagnose. Endvidere omtales Pneumococcernes Variation foraarsaget af ugunstige Forhold hos Omgivelserne. Pneumococcerne kan miste Virulensen og Evnen til at danne Kapsel, hvorved Typespecificiteten tabes, men Artsspecificiteten bevares. En virulent, kapselholdig, typespecifik Pneumococ betegnes S Form, en avirulent, kapselløs, ikke typespecifik Pneumococ betegnes R Form.

Pneumococcernes Variation er særlig undersøgt ved Dyrkning i homologt Immunserum (*Stryker* (84), *Griffith* (37), *Reimann* (75-76), *Dawson* (19-20) og andre). Koncentreret Serum er ikke nødvendig for at faa Pneumococcerne dissocieret, en Serumfortynding paa 1 : 256 er tilstrækkelig til at fremkalde en delvis Forandring af S Pneumococcer til R Pneumococcer, men den bedste Koncentration er 10-25 %. Reversionen af R til S lykkes in vitro ved Dyrkning i 10 % Anti-R-Serum og in vivo ved subcutan Injektion paa hvide Mus af store Mængder levende R Kultur eller smaa Mængder R Kultur sammen med store Mængder varmedræbt homolog S Kultur.

Endvidere omtales Pneumococcernes Omdannelse fra een Type til en anden Type, som først var meddelt af *Griffith* (38) og senere af *Reimann* (77), *Dawson* (21), *Baurhenn* (8), *Alloway* (1-2) og andre. *Griffith* var den første for hvem Omdannelsen lykkedes in vivo, idet han sprøjtede R Pneumococcer fra een Type subcutant paa Mus sammen med varmedræbte S Pneumo-

coccer fra en anden Type. For *Dawson & Sia* (25-26, 80) lykkedes Typeomdannelsen in vitro, idet de i Reagensglas med varmedræbte S Pneumococcer, Anti-R-Serum og Blodbouillon udsaaede minimale Mængder R Kultur. For *Alloway* (1-2) lykkedes Omdannelsen ved i Stedet for S Vaccine at anvende filtreret Extrakt af Pneumococcerne.

Endelig omtales forskellige Forfatteres Undersøgelser over Temperaturens Indflydelse paa Pneumococcer, og de fleste (*Lange* (54), *Paul* (74), *Avery* (6), *Stryker* (84), *Bauhenn* (8), *Griffith* (38), *Reimann* (77), *Dawson* (20-21) og mange andre) mener, at Pneumococcerne bliver dræbt ved Opvarmning til 60° i 20 Minutter, medens de færreste (*Haendel & Lange* (40), *Dunlop* (29)) mener, at længere Tids Opvarmning til 60° eller højere Temperatur end 60° maa anvendes for at være sikker paa, at alle Pneumococcerne bliver dræbt.

II. Afsnit. Egne Forsøg.

Kapitel I omtaler det rent tekniske til Forsøgene, saaledes de anvendte Næringssubstrater til Undersøgelse af Vaccinens Sterilitet, de anvendte Kulturer, Bestemmelse af de kapselholdige Pneumococcers Virulens overfor hvide Mus, Fremstillingen af S og R Vaccine, Pneumococcernes specifikke opløselige Substans, Immunserum og R Pneumococcer; Udførelsen af in vitro og in vivo Sterilitetsprøverne, Omdannelsesforsøgene in vivo, som blev udført paa samme Maade som meddelt af *Griffith* (38), Udførelsen af M&B Forsøgene, Forgæringsforsøgene og Filtrationsforsøgene, Omdannelsesforsøgene in vitro, som blev udført paa samme Maade som angivet af *Dawson & Sia* (25-26) og Udførelsen af Absorptionsforsøgene.

Kapitel II handler om Undersøgelserne over R Pneumococcer. Ved Dyrkning i homologt Immunserum danner alle S Pneumococcer ikke lige let stabile R Pneumococcer, d. v. s. R Pneumococcer, som ikke reverterer til homologe S Pneumococcer ved Dyrkning i Serumbouillon eller ved intraperitoneal Injektion paa Mus, saaledes var R 8, R 22 og R 35 dannet af den homologe

S Pneumococ efter henholdsvis 83, 20 og 20 Passager ikke stabile, hvorimod R 1 og R 2 allerede var stabile efter 5 Passager af S 1 og S 2 i Immunserum. R Pneumococcerne forholdt sig paa samme Maade overfor M&B som de homologe S Pneumococcer; var disse resistente vilde R Formerne ogsaa være resistente, var de følsomme, var R Formerne ogsaa følsomme. Alle undersøgte R Pneumococcer var serologisk identiske, og de nydannede S Pneumococcers R Former var identiske med de i Forsøget anvendte R Former. Identiteten blev bevist ved krydsvis Absorption med de forskellige R Pneumococcer. Endvidere bevises det, at Anti-R-Serum tømmes for Agglutininer ved Absorption baade med de homologe R og S Pneumococcer og de heterologe R Pneumococcer, medens Anti-S-Serums Agglutininer kun fjernes ved Absorption med de homologe S Pneumococcer, men ikke med R Pneumococcer, idet disse kun kunde fjerne de artsspecifikke Agglutininer og ikke de typespecifikke.

Kapitel III omtaler Undersøgelser over Vaccinens Sterilitet. Pneumococcer, som voksede i æggehvideholdige Substrater, skulde opvarmes til højere Temperatur, for at alle skulde blive dræbt, end Pneumococcer, som blev dyrket i Substrater, der ikke indeholdt Serumprotein. Jo tættere Suspensionen af Pneumococcer var, desto højere Temperatur krævedes der for at dræbe alle Pneumococcerne; men i intet Tilfælde dyrkedes Pneumococcer efter Opvarmning af Kulturerne til 60° i 20 Minutter. Selve Vaccinen til Omdannelsesforsøgene var fremstillet efter Opvarmning til 60° i 20 Minutter. 2917 Sterilitetsforsøg in vitro og 533 Sterilitetsforsøg in vivo blev udført paa Vaccinen. In vitro Forsøgene blev foretaget ved Udsaaning af forskellige Mængder Vaccine i og paa forskellige flydende og faste Næringssubstrater, som blev stillet aerobt, anaerobt eller i CO_2 Atmosfære, in vivo Forsøgene ved subcutan Injektion paa hvide Mus enten af Vaccine alene eller sammen med andre Bakterier (Coli, Streptococcer, Staphylococcer, dræbte R Pneumococcer), Pneumococcernes specifikke, opløselige Substans (SSS) eller paa Mus, som var forbehandlet med Mucin eller Alkohol.

Kapitel IV handler om alle Omdannelsesforsøg in vivo. Alle Museforsøgene blev foretaget ved subcutant at injicere smaa Mængder R Kultur af een Type sammen med store Mængder varmedræbte S Pneumococcer (Vaccine) af en anden Type paa hvide Mus. Ved at foretage Forsøgene efter *Griffith's* Metode lykkedes Omdannelsen fra Type 1 til Typerne 2 og 35, men ikke til Typerne 8, 14, 20 og 22. Den nydannede Type 35 havde samme Virulens som Vaccinens Type 35 og ikke som Type 1, hvorfra den var dannet.

For at bevise, at de nydannede Pneumococcer bestod af samme Corpus som de injicerede R Pneumococcer, anvendtes markerede Stammer, d. v. s. Stammer med kendte konstante Egenskaber knyttet til Corpus, saasom Kemoresistens og Forgæringsevne. Disse 2 Egenskaber blev ikke paavirket af Opbevaring, Musepassager eller Kulturpassager i Serumbouillon. Ved at injicere en kemoresistent R Form af een Type sammen med følsom Vaccine S Form af en anden Type dyrkedes fra Musen kemoresistente S Former af Vaccinens Type. For at udelukke, at de følsomme S Pneumococcer under Opvarmning blev resistente, blev Forsøget vendt om, idet den følsomme R Form blev injiceret sammen med den kemoresistente S Form, og nu dyrkedes fra Musene følsomme S Former af Vaccinens Type. Paa denne Maade lykkedes Forsøgene: R 1 føl. + Vaccine S 2 res. → Type 2 føl.; R 1 res. + Vaccine S 2 føl. → Type 2 res.; R 2 føl. + Vaccine S 1 res. → Type 1 føl.; R 2 res. + Vaccine S 1 føl. → Type 1 res. og R. 1 føl. + Vaccine S 8 res. → Type 8 føl.

Ved at injicere en R Form af een Type sammen med en S Form af en anden Type, hvor R Formen havde en Forgæringsevne, der var forskellig fra S Formen, dyrkedes kapsulate Pneumococcer fra Musen af samme Type som Vaccinens S Form, men med R Formens Forgæringsevne. Saaledes havde de nydannede Typer 2, 6, 8 og 35 samme Forgæringsevne som de injicerede R 1 Pneumococcer og ikke som Vaccinens Type. En nydannet Type 2's R Form havde samme Forgæringsevne som den til Forsøget anvendte R 1 og ikke som Vaccine-Typens R Form (R 2).

Forsøgene med Pneumococcer, der havde forskellig Kemo-

resistens og Forgæringsevne, viste, at den nydannede Type bestod af Corpus fra den ene Type og Kapslen fra den anden Type og havde den ene Types Corpusegenskaber og den anden Types Kapselegenskaber.

De nydannede Typers Kolonier paa Blodagarplade lignede i de fleste Tilfælde Vaccine-Typens Kolonier, men nogle af Kolonierne var smaa og umulige at skelne fra R Kolonier. Med Immunserum var Kapselsvulsten som Regel tydelig, men nogle af de nydannede Pneumococcer gav kun svag Kapselsvulst, som ikke blev bedre ved at lade Pneumococcerne gaa gennem Mus. Hos samme Forsøgsmus fandtes Kapslen hos nogle af de nydannede Typer kun let fortykket, hos andre kun svagt udviklet og hos andre igen fuldt udviklet. Kapselsvulsten var hos nogle Pneumococcer ujævn, idet der ligesom fandtes »Buler« paa den. Hos nogle af Forsøgsmusene havde en Del af R Pneumococcerne taget heterologe Kapsler, andre homologe Kapsler, og andre forblev R Form.

Forsøgene viste, at ved Anvendelse af unge R Pneumococcer, d. v. s. R Former dannet af de homologe S Former efter faa Passager i Immunserum, lykkedes Omdannelsen fra en Type til en anden Type lettere (gennemsnitlig i 32,5 % af Musene) end ved Anvendelse af gamle R Pneumococcer, d. v. s. R Former dannet af de homologe S Former efter mange Passager i Immunserum (gennemsnitlig i 4,4 % af Musene).

Pneumococcernes Forgæringsevne blev undersøgt overfor Arabinose, Xylose, Dulcit, Inosit, Glukose, Galaktose, Laktose, Sakkarose, Inulin, Mannit, Melibiose og Salicin. De 4 første Stoffer forgæredes aldrig, de næste 4 altid, undtagen af en bestemt Type 11, og de 4 sidste Stoffer forgæredes forskelligt af de forskellige Pneumococcer. Forsøget viste, at serologisk forskellige Typer ikke adskilte sig tydeligt fra hinanden med Hensyn til Forgæring, at serologisk identiske Stammer ikke altid havde samme Forgæringsevne, og at R Pneumococcerne i det væsentlige forgærede som de homologe S Pneumococcer.

Endelig omtales i Kapitlet enkelte Forsøg paa at fremstille det hypotetiske, termolabile Stof, som er i Stand til at paahæfte Kapselsubstansen fra en Type til Corpus af en anden Type. 100 Mus blev sprøjtet subcutant med R 1 Kultur, Vaccine S 2 (frem-

stillet ved Autoklavering i 1 Time ved 100°) og Filtratet af Pneumococcer Type 2 Kultur, men hos ingen Mus lykkedes Omdannelsen fra Type 1 til Type 2.

Kapitel V handler om Omdannelsesforsøgene in vitro. Der blev ialt foretaget 787 Reagensglasforsøg paa samme Maade som angivet af *Dawson & Sia* (25-26). Forsøgene blev foretaget ved at tilsaa Reagensglas indeholdende Næringssubstrat, Vaccine og Anti-R-Serum med R Kultur, stille Blandingen i Termostat ved 37° og enten udsaa daglig i de følgende 15 Dage paa Blodagarplade og i Serumbouillon til Undersøgelse for kapsulate Pneumococcer eller Dagen efter omsaa i Reagensglas med samme Mængde Næringssubstrat, Vaccine og Anti-R-Serum, udsaa paa Blodagarplade og i Serumbouillon og fortsætte saaledes i de følgende 4-10 Dage. Næringssubstratet var som Regel Blodbouillon, enkelte Gange Serumbouillon, Vaccinen var Centrifugatet fra Oxehjertebouillon- eller Serumbouillonkultur opvarmet til 60° i 20 Minutter. Anti-R-Serum med en Titer paa 640-1280 blev tilsat Reagensglassene i forskellig Mængde (0,1-0,3 cm^3), af forskellig Koncentration fra ufortyndet Serum til Fortyndingen 1 : 400. Mængden af tilsatte R Pneumococcer varierede ligeledes, idet 1 Draabe af forskellige Fortyndinger fra 10^{-9} til Fuldkultur blev tilsat hvert Reagensglas. I ingen af mine Forsøg (fra Type 1 til Typerne 2, 8, 22 og 35 og fra Type 2 til Type 1) lykkedes Omdannelsen.

Kapitel VI omtaler serologiske Undersøgelser over de nydannede Typer og deres Konstans. Ved krydsvis Absorption vises, at den nydannede Type 2 dannet af Type 1 er serologisk identisk med den almindelige Type 2, ligeledes den nydannede Type 8 med den almindelige Type 8 og den nydannede Type 35 med den almindelige Type 35. Ved Opbevaring og ved talrige Dyrkninger og Musepassager bevarede de nydannede Pneumococcer deres erhvervede Egenskaber baade i Kapsel og Corpus.

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